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PRINCIPAL INVESTIGATOR: Mark F. Bear, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts Institute of Technology
Cambridge, MA 02139

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Mark F. Bear, Ph.D.

E-Mail: mbear@mit.edu

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14. ABSTRACT

Purpose: The goal of this project is to determine the underlying synaptic dysfunction in Tuberous Sclerosis Complex (TSC). Scope: TSC is a multi-system genetic disorder with central nervous system dysfunction as a defining factor. The most common clinical features are mental retardation, epilepsy, autism, anxiety and mood disorders. Fragile X syndrome (FXS), another form of inherited mental retardation and autism, shares many of the same molecular and clinical features as TSC. Much of the pathophysiology in FXS can be ameliorated through modulation of Group 1 metabotropic glutamate receptors (mGluRs). Since the two disorders share key features suggests that TSC and FXS may also share common pathogenic mechanisms. Therefore, we tested whether altered synaptic protein synthesis, plasticity and hippocampal-dependent behavior could be ameliorated through modulation of mGluR function in a mouse model of TSC. Major findings: Unlike in FXS where negative modulation of mGluR function has proven beneficial, we found that augmenting mGluR function through application of a mGluR5 positive allosteric modulator (PAM) ameliorates several of the synaptic and behavioral deficits observed in a mouse model of TSC. Significance: These results suggest that direct modulation of mGluR activity with PAMs may serve as a therapeutic intervention for the treatment of TSC.

15. SUBJECT TERMS
autism, Tuberous Sclerosis Complex, Fragile X Syndrome, metabotropic glutamate receptor, rapamycin, positive allosteric modulator, long-term depression, synaptic protein synthesis

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Introduction:

Tuberous sclerosis complex (TSC) is a multi-system genetic disorder that affects roughly 1 in every 6000 individuals. The disorder is characterized by benign tumors in multiple organs, including the brain. Central nervous system dysfunction is a defining factor in the disorder, with the some of the most common clinical features being mental retardation, epilepsy, autism, anxiety and mood disorders (Prather and de Vries, 2004). Fragile X syndrome (FXS), another form of inherited mental retardation and autism, shares many of the same clinical features as TSC. Moreover, there are biochemical interactions between FMRP, the protein disrupted in FXS, and the TSC1/TSC2 complex (Narayanan et al., 2007). A leading theory on the etiology of FXS holds that exaggerated protein synthesis linked to activation of Gp1 mGluRs (mGluRs 1 and 5) results in changes in synaptic structure and function that are the root cause of cognitive impairment. Indeed, genetic reduction and pharmacological inhibition of mGluR5 has been shown to correct almost every phenotype examined in the *Fmr1* KO mouse (Dolen et al., 2007; Michalon et al., 2012). That there are commonalities between TSC and FXS, ranging from the molecular to clinical level, suggests that TSC and FXS may share common pathogenic mechanisms. This inspired us to perform the complimentary experiments used to characterize FXS in a well-established animal model of TSC, the *Tsc2*$^{+/-}$ mouse. Unexpectedly, we found a reduction in mGluR-dependent synaptic plasticity and protein synthesis in the hippocampus of the Tsc2$^{+/-}$ mice. We hypothesize that these alterations in mGluR-dependent plasticity and protein synthesis are causally related to the cognitive dysfunctions seen in TSC and that *augmenting* mGluR5 function with mGluR5 positive allosteric modulators (PAMs) would ameliorate the synaptic and behavioral deficits seen in *Tsc2*$^{+/-}$ mice. Corroborating the efficacy of mGluR5 PAM treatment at the behavioral level is an essential step in validating this intervention as a putative therapy for TSC. Therefore, we propose to fully characterize synaptic and behavioral impairments in *Tsc2*$^{+/-}$ mice and to determine the effectiveness of mGluR PAM treatment on these deficits. The specific aims of this proposal are:

<u>Specific aim 1:</u> Comparison of mGluR5 PAM and rapamycin treatment on hippocampal protein synthesis and hippocampal-dependent behavioral deficits in *Tsc2*$^{+/-}$ mice.

<u>Specific aim 2:</u> Characterization of visual cortical plasticity in *Tsc2*$^{+/-}$ mice and comparison of mGluR5 PAM and rapamycin treatment.

<u>Specific aim 3:</u> Characterization of higher cognitive function in *Tsc2*$^{+/-}$ mice.

Body:

Since the initiation of this project on April 1 2011, we have made considerable progress in the proposed research. We have completed the majority of experiments outlined in the first year of our approved Statement of Work and have initiated the behavioral experiments proposed in Aim 3. The progress made on this project to date has culminated in several presentations at scientific meetings and a peer-reviewed article describing our findings has recently been published in the journal *Nature* (Auerbach et al., 2011).

Specific aim 1: Comparison of mGluR5 PAM and rapamycin treatment on hippocampal protein synthesis and hippocampal-dependent behavioral deficits in *Tsc2*$^{+/-}$ mice.

Rationale:

TSC is caused by an inactivating mutation in either the TSC1 or TSC2 gene. Normally, one of the major cellular functions of the TSC1/TSC2 protein complex is to limit protein synthesis by inhibiting Rheb, a Ras family GTPase (Kwiatkowski and Manning, 2005). Rheb and its downstream effector, mTOR, act as master regulators of protein synthesis and cell growth. The regulation of protein synthesis is necessary for many functions in the brain and body. In particular, the long-term maintenance of synaptic plasticity requires the synthesis of new proteins. The persistent modification of synaptic strengths is thought to be the neural basis of learning and memory, and the improper regulation of synaptic protein synthesis leads to altered synaptic plasticity and adversely affects learning, memory, and cognition (Ozonoff et al., 2008). Our hypothesis is that dysregulated synaptic protein synthesis is a determining factor in the learning and cognitive deficiencies seen in TSC.

Group 1 metabotropic glutamate receptors (Gp1 mGluRs) are widespread and potent regulators of local synaptic protein synthesis (Job and Eberwine, 2001; Weiler and Greenough, 1993) and mGluR-dependent translation has been shown to play a major role in many forms of synaptic plasticity, including a type of long term depression (termed mGluR-LTD) in the hippocampus, an area of the brain known to be important for learning and memory. This form of LTD is dependent on mGluR5 activation and requires rapid protein synthesis (Huber et al., 2000; Huber et al., 2001). It is important to note that both mGluR-LTD and basal protein synthesis are exaggerated in the hippocampus of a mouse model of FXS, another form of inherited mental retardation and autism (Huber et al., 2002; Osterweil et al., 2010). A leading theory on the etiology of FXS holds that exaggerated protein synthesis linked to Gp1 mGluR activation results in changes in synaptic structure and function that are the root cause of cognitive impairments (Bear et al., 2004).

Previously, we demonstrated that there is disrupted mGluR function in the hippocampus of *Tsc2*$^{+/-}$ mice. Specifically, we found a reduction in mGluR-LTD and an accompanying decrease in basal protein synthesis in this region, with no change in NMDA-receptor dependent LTD (See Figures 1 & 2). Furthermore, treatment with both mGluR5 PAM and rapamycin, an inhibitor of mTORC1, reversed the electrophysiological impairments observed in *Tsc2*$^{+/-}$ mice (See Figure 3). Since alterations in hippocampal function have adverse effects on learning and cognition, deficits of this kind are likely to contribute to the cognitive impairments seen in TSC. Therefore, it is important to fully characterize the nature of the relationship between Rheb/mTOR signaling, mGluR-dependent plasticity, and the electrophysiological and behavioral impairments seen in *Tsc2*$^{+/-}$ mice. In the experiments proposed under this aim, we directly compared the effect of rapamycin and mGluR5 PAM treatments on hippocampal protein synthesis and determined the extent to which mGluR5 PAM treatment can reverse the behavioral impairments previously shown to be rescued by rapamycin in *Tsc2*$^{+/-}$ mice (Ehninger et al., 2008).

Task 1. Measure the effect of mGluR PAM and rapamycin treatment on protein synthesis rates in hippocampus of *Tsc2*$^{+/-}$ mice (estimate use of 50 mice) (timeframe, months 1-3).

It has been shown that mGluR-LTD requires the rapid synthesis of new proteins (Huber et al., 2000). In *Fmr1* KO mice, it is believed that this excessive protein synthesis serves as the cellular mechanism which leads to the exaggeration of mGluR mediated processes, including mGluR-LTD (Bear et al., 2008). Therefore, we hypothesized that decreased basal protein synthesis levels may account for the deficient mGluR-LTD seen in *Tsc2*$^{+/-}$ mice and, as predicted, basal protein synthesis rates are in fact reduced in *Tsc2*$^{+/-}$ mice (See Figure 2). This finding suggested the possibility that protein(s) required for mGluR-LTD are deficiently translated in the hippocampus of *Tsc2*$^{+/-}$ mice. Therefore, under this aim we performed additional experiments examining the levels of Arc, a plasticity-related protein that is rapidly synthesized in response to mGluR activation and is required for mGluR-LTD (Park et al., 2008; Waung et al., 2008). Interestingly, we found that Arc expression is indeed decreased in *Tsc2*$^{+/-}$ hippocampal slices (See Figure 4A). To determine whether this decrease was due to diminished translation, we measured the amount of newly synthesized Arc in *Tsc2*$^{+/-}$ mice by performing immunoprecipitation experiments on metabolically labeled slices (Osterweil et al., 2010). Examination of the ^{35}S-incorporated fraction revealed a significant reduction in Arc translation in the hippocampus of *Tsc2*$^{+/-}$ mice (See Figure 4B). These results strongly suggest that mGluR-LTD is deficient in the *Tsc2*$^{+/-}$ hippocampus because of a decrease in the translation of proteins required to stabilize LTD, including Arc.

To test the hypothesis that the deficient basal protein synthesis observed in *Tsc2*$^{+/-}$ mice is a specific consequence of unregulated mTOR activity, we first examined the effects of the mTORC1 inhibitor rapamycin. We found that acute rapamycin treatment (20 nM) restored protein synthesis levels in the *Tsc2*$^{+/-}$ mice to WT levels, whereas this same treatment had no effect on slices from WT mice (Figure 5). These findings are entirely consistent with our previous observations demonstrating that acute rapamycin treatment restores mGluR-LTD in *Tsc2*$^{+/-}$ mice to WT levels (Figure 3). Moreover, the rescue of mGluR-LTD in *Tsc2*$^{+/-}$ mice appears to be specifically due to recovery of the protein-synthesis-dependent component of mGluR-LTD, as the effect of rapamycin in *Tsc2*$^{+/-}$ slices is eliminated in the presence of the protein synthesis inhibitor cycloheximide (Figure 3).

A model that best fits these data is that unregulated mTOR activity caused by the *Tsc2*$^{+/-}$ mutation suppresses the protein synthesis that is required for mGluR-LTD (Figure 7A)). In the mouse model of FXS, excessive mGluR-LTD and hippocampal protein synthesis can be corrected by reducing signaling through mGluR5 activation (Dolen et al., 2007; Osterweil et al., 2010). We therefore determined if the opposite approach of potentiating mGluR5 signaling with a positive allosteric modulator (PAM) could be beneficial in this model of TSC. PAMs are compounds that do not activate mGluR5 directly but act on an allosteric site to potentiate physiological activation of the receptor (Conn et al., 2009). Indeed, we found that pretreatment of hippocampal slices with the mGluR5 PAM 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB;(Kinney et al., 2005)) restored the magnitude of mGluR-LTD in *Tsc*2$^{+/-}$ mice to WT levels (Figure 3). The rescue of LTD appears to be due specifically to recovery of the protein-synthesis-dependent component because the effect of CDPPB was eliminated by cycloheximide (Figure 3). Consistent with this conclusion, CDPPB treatment also restored basal protein synthesis levels (Figure 5) and rescued the deficit in Arc synthesis in the *Tsc*2$^{+/-}$ mice (Figure 5). Thus, allosteric augmentation of mGluR5 signaling can overcome the inhibitory effect of unregulated mTOR activity on the synaptic protein synthesis that supports LTD.

Task 2. Characterization of contextual fear conditioning and effect of mGluR5 PAM treatment (estimate use of 150 mice) (timeframe, months 4-9).

We have shown that there is disrupted mGluR function in the hippocampus of *Tsc2*$^{+/-}$ mice. The hippocampus is an area of the brain known to be vital for many forms of learning and memory (Eichenbaum, 2004). Alterations in hippocampal function have adverse effects on learning and cognition, and therefore are likely to contribute to the cognitive impairments seen in TSC. Therefore, it is imperative to determine if mGluR5 PAM treatment is effective at reversing hippocampal-dependent deficits in *Tsc2*$^{+/-}$ mice. Previously, it has been demonstrated that rapamycin treatment reverses the deficits in hippocampal-dependent learning observed in these mice (Ehninger et al., 2008). Therefore, it is important to fully characterize the nature of the relationship between mTOR signaling, mGluR-dependent plasticity, and the electrophysiological and behavioral impairments seen in *Tsc2*$^{+/-}$ mice. Under this aim, we sought to determine if mGluR5 PAM treatment can reverse the behavioral impairments previously shown to be rescued by rapamycin in *Tsc2*$^{+/-}$ mice.

2a. Development of context discrimination fear conditioning paradigm and confirmation of the ability of WT mice to reliably distinguish context in this behavioral paradigm (months 4-6).

In a recent study, cognitive impairments in *Tsc2*$^{+/-}$ mice were shown to be significantly improved by treating the animals with the mTORC1 inhibitor rapamycin (Ehninger et al., 2008). In light of our previous electrophysiological and biochemical findings, we wondered if a similar amelioration of the behavioral deficits observed in *Tsc2*$^{+/-}$ mice would be observed by administering the mGluR5 PAM. Enhinger et al. (2008) reported an impairment in the ability of *Tsc2*$^{+/-}$ mice to distinguish between familiar and novel contexts in a fear conditioning task. Advantages of this task are that the learning occurs in one trial, making it amenable to acute drug treatment, and the memory is hippocampus dependent (Frankland et al., 1998), and requires both mGluR5 activation (Lu et al., 1997) and new protein synthesis at the time of training (Steidl and Yool, 1999). In this assay, mice are first exposed to a distinctive context in which they receive an aversive foot shock. The next day, context discrimination is tested by dividing the animals into two groups: one is placed in the familiar context associated with the shock; the other is placed in a novel context (see Figure 6, and Appendix for detailed methods). Context discrimination is assessed by measuring the amount of time the animals express fear by freezing in each context. As one would expect, more freezing is observed by animals introduced into the familiar context, where they previously experienced the foot shock, compared to the animals introduced into the novel context. Initially we determined whether WT mice could discriminate familiar from novel contexts and whether the quality of this behavior was comparable to that previously reported. Figure 6 demonstrates that, indeed, WT mice clearly discriminate between the two contexts as they spent significantly more time freezing in the familiar (50.0 ± 7.7%) versus the novel context (34.1 ± 3.2%, P = 0.003). These findings are consistent with those previously reported by Ehinger et al, (2008) (Ehninger et al., 2008).

2b. Replication of context discrimination phenotype in *Tsc2*$^{+/-}$ mice (months 4-6).

A recent study has demonstrated that *Tsc2*$^{+/-}$ mice trained in the context fear conditioning task have a deficit in their ability to discriminate between the familiar context and a novel context (Ehninger et al., 2008). Under this sub-aim we have replicated this phenotype. Unlike the WT animals described above, *Tsc2*$^{+/-}$ mice did not demonstrate the ability to discriminate between the familiar and novel contexts as they spent a comparable amount of time freezing in both contexts

(familiar: 40.9 ± 5.3%; novel, 39.3 ± 5.2%; P = 0.501; Figure 6B). These findings are significant as they demonstrate that this behavioral assay is a sensitive measure of disruptions of a hippocampal-dependent process.

2c. Detemine if mGluR PAM treatment can rescue context discrimination phenotype in *Tsc2*$^{+/-}$ mice (months 7-9).

Enhinger et al. (2008) demonstrated that *Tsc2*$^{+/-}$ mice showed a deficit in context fear-conditioning and that rapamycin treatment rescued this deficit. While rapamycin has been used clinically, it is not an ideal drug for the chronic treatment of TSC due to its strong immunosuppressive properties. We believe that direct modulation of mGluR activity with the use of PAMs is a novel therapeutic strategy for the treatment of TSC that would not share these detrimental properties. Although we have shown that both mGluR5 PAM and rapamycin treatment have similar effects on the electrophysiological impairments in *Tsc2*$^{+/-}$ mice, it is important to determine if these similarities extend to the behavioral level. Therefore, under this sub-aim we measured the effect of mGluR5 PAM treatment on the context discrimination deficits observed in *Tsc2*$^{+/-}$ mice. To test the effect of augmenting mGluR5 signaling, WT and *Tsc2*$^{+/-}$ mice were injected intraperitoneally with CDPPB (10 mg/kg) 30 min before training. Although this treatment had no effect on behavioral performance in WT mice (familiar: 42.3 ± 3.7%, novel: 26.4 ± 3.6%, P = 0.005, Figure 6B), it was sufficient to correct the deficit in context discrimination observed in *Tsc2*$^{+/-}$ mice (familiar: 44.5 ± 4.3%, novel: 31.6 ± 3%, P = 0.034, Figure 6B). These results show that augmentation of mGluR5 signaling is beneficial at the behavioral level in *Tsc2*$^{+/-}$ mice and that disrupted mGluR5 function may be relevant to cognitive impairments associated with TSC.

Our data demonstrate that mutations causing FXS and TSC, two disorders associated with autism and intellectual disability, show mirror symmetrical alterations in protein-synthesis-dependent LTD and have beneficial responses to treatments that modulate mGluR5 in opposite directions (Figure 7A). Since these two mutations cause opposite alterations in mGluR-LTD and protein synthesis, this raised the intriguing possibility that these two mutations could cancel one another on this functional axis. In order to test this hypothesis, in an additional series of experiments, we introduced an *Fmr1* deletion into the *Tsc2*$^{+/-}$ background by crossing *Tsc2*$^{+/-}$ males with *Fmr1*$^{+/-}$ females (Figure 7B). This approach enabled us to compare directly with WT the effects of the *Tsc2*$^{+/-}$ and *Fmr1*$^{-/}$*y* mutations in littermates reared under identical conditions. Interestingly, when we compared context discrimination in the *Tsc2*$^{+/-}$ and *Fmr1*$^{-/}$*y* mice we found that that they both share a deficit in this measure of memory (Figure 7E). Remarkably, this memory deficit was erased in the double mutants. In an additional set of electrophysiological experiments we tested whether a similar rescue of mGluR-LTD was observed. We found that mGluR-LTD was diminished in *Tsc2*$^{+/-}$ mice and excessive in the *Fmr1*$^{-/}$*y* mice, compared with WT. However, mice harboring both mutations showed mGluR-LTD that was indistinguishable from WT (Figure 7C,D). These results suggest that the opposing synaptic deviations seen in *Tsc2*$^{+/-}$ and *Fmr1*$^{-/}$*y* mice manifest similarly at the behavioral level, and introducing both mutations not only reverses the disruptions of synaptic plasticity but rescues this memory impairment as well.

Having found a robust phenotype in an assay of hippocampal-dependent function using the context discrimination paradigm, we chose not to pursue an investigation determining the effects of mGluR5 PAM treatment on Morris water maze performance as little additional insight would be gained. We instead focused our attention on characterizing higher cognitive function in *Tsc2*$^{+/-}$ mice (see below).

Specific aim 3: Characterization of higher cognitive function in *Tsc2*$^{+/-}$ mice.

The cognitive manifestations of TSC are complex and diverse. However, the cognitive impairments most consistently associated with the disorder are deficits in executive-attentional function (Gillberg et al., 1994; Prather and de Vries, 2004). Executive function is an umbrella term used to describe a set of cognitive skills involved in attention, inhibitory control and cognitive flexibility. Despite extensive clinical research into this core deficit in TSC, impairments in executive function have received surprisingly little attention in pre-clinical research. In particular, there has been no published research exploring executive function in animal models of TSC. Here, we propose to establish a battery of behavioral measures to assess executive function in *Tsc2*$^{+/-}$ mice, and to subsequently utilize this battery to investigate the role of metabotropic glutamate receptor signaling in this core deficit.

Having demonstrated that the hippocampal-dependent deficit in contextual fear conditioning is ameliorated by both pharmacological facilitation of mGlur5 function with PAMs and genetic intervention, we have focused our efforts on determining whether higher cognitve functions are compromised in the *Tsc2*$^{+/-}$ mouse utilizing the five choice serial reaction time task (5CSRTT), which is a well-established operant conditioning paradigm used to assess executive function in rodents. To this end, we have successfully initiated a partial food deprivation paradigm in both WT and *Tsc2*$^{+/-}$ mice, with no apparent adverse effects in either group of animals. Additionally, both groups of animals are currently undergoing daily habituation sessions in the behavioral testing apparatus in order that we may proceed with the experiments proposed under this aim.

Key Research Accomplishments:

1. Demonstrated that mGluR5 PAM and rapamycin treatment reverse deficits in hippocampal protein synthesis.
2. Provided evidence that mGluR-LTD is deficient in the *Tsc2*$^{+/-}$ hippocampus because of a decrease in the translation of proteins required to stabilize LTD, including Arc.
3. Replicated previously published findings demonstrating that WT mice can discriminate familiar from novel contexts and that the quality of this behavior was comparable to that previously reported.
4. Demonstrated that the *Tsc2*$^{+/-}$ show a deficit in hippcompal-dependent context discrimination.
5. Demonstrated that mGluR5 PAM and rapamycin treatment reverses hippocampal dependent behavioral deficits in *Tsc2*$^{+/-}$ mice.
6. Demonstrated the feasibility of crossing two mouse models of Autism can restore normal synaptic and behavioral function.

Reportable Outcomes:

Findings

- Demonstrated that synaptically-induced mGluR-LTD is also deficient in *Tsc2*$^{+/-}$ while mechanistically distinct NMDAR-LTD is unaffected
- Demonstrated that deficit in hippocampal protein synthesis in *Tsc2*$^{+/-}$ mice can be reversed by rapamycin and mGluR5 PAM treatment

- Demonstrated that the protein Arc, known to be required for mGluR-LTD, is deficiently translated in *Tsc2*$^{+/-}$ mice, suggesting a causal relationship between deficit in synaptic plasticity and protein synthesis
- Demonstrated that deficient Arc translation is rescued by mGluR5 PAM
- Replicated previous results of hippocampal-dependent behavioral abnormalities in *Tsc2*$^{+/-}$ mice
- Demonstrated that deficits in hippocampal-dependent behavior can be reversed by mGluR5 PAM
- Demonstrated the feasibility of crossing two mouse models of Autism can restore normal function.
- Replicated previous results of exaggerated mGluR-LTD in Fmr1 KO mice
- Demonstrated novel behavioral deficits in *Fmr1*$^{-/y}$ mice that is shared with *Tsc2*$^{+/-}$ mice, suggesting opposite synaptic deficits can lead similar cognitive impairments.
- Demonstrated that introducing both *Tsc2* and *Fmr1* mutations into mice reverses deficits seen in single mutants at biochemical, electrophysiological, and behavioral levels.

Publications

- Published results as article in the journal Nature: Auerbach BD, Osterweil EK, Bear MF. Mutations causing syndromic autism define an axis of synaptic pathophysiology. Nature. 2011 Nov 23;480(7375):63-8.

Talks & Presentations

- Keynote speaker at the Cold Spring Harbors Conference: Synapses - From Molecules to Circuits & Behavior, Cold Spring Harbor, NY, April, 2011. (Mark Bear)
- Presented at the Cold Spring Harbors Conference: Synapses - From Molecules to Circuits & Behavior, Cold Spring Harbor, NY, April, 2011. (Benjamin Auerbach)
- Swammerdam Lecture, Neuroscience Graduate School Seminar Series, University of Amsterdam, April 19, 2011. (Mark Bear)
- Presented at the Pediatric Academic Societies Annual Meeting, Session 3110: Psychopharmacologic treatment of intellectual, Denver, May, 2011. (Mark Bear)
- Presented at the Society of Biological Psychiatry Annual Meeting, San Francisco, May, 2011. (Mark Bear)
- Invited seminar at University of Copenhagen 4th Annual Midsummer Lecture, July, 2011. (Mark Bear)
- Presented at the International TSC Research Conference: Summit for Drug Discovery in TSC and Related Disorders, Washington DC, July, 2011. (Benjamin Auerbach)
- Keynote Speaker at FRAXA Investigators Meeting, Southbridge, MA, September, 2011. (Mark Bear)
- Presented at the 7th International metabotropic glutamate receptor conference, Catania, IT, October, 2011. (Mark Bear, Benjamin Auerbach)
- Presented at the "Howard Hughes Medical Institute Investigator's Meeting, Washington, DC, November, 2011. (Mark Bear, Benjamin Auerbach)
- Presented at Cell Symposia: Autism Spectrum Disorders: From Mechanisms to Therapies, November, 2011. (Mark Bear)
- Invited seminar at the Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA, December, 2011. (Benjamin Auerbach)
- Invited seminar at the Department of Physiology and Biophysics, University at Buffalo, Buffalo, NY, February, 2012. (Benjamin Auerbach)
- Presented at NIH/NINDS Grand Rounds Seminar Series, Washington, DC, March, 2012. (Mark Bear)

- Presented at the Developmental Cognitive Neuroscience Symposium, Brain and Cognitive Sciences Department, Massachusetts Institute of Technology, Cambridge, MA, March, 2012. (Benjamin Auerbach)
- Invited seminar at Baylor College of Medicine, Jan and Dan Duncan Neurological Research Institute, Houston, TX, April, 2012. (Mark Bear)
- Ray Fuller Lecture, American Society for Pharmacology and Experimental Therapeutics Annual Meeting, San Diego, CA, April, 2012. (Mark Bear)

Conclusion:

Tuberous sclerosis complex (TSC) is a debilitating disorder that leads to significant disability for individuals and disrupt the lives of their families. Central nervous system disruption is one of the most pronounced features of the disorder, yet there is no treatment directed at the core neurological dysfunction that is definitive of TSC. In this research project, we have shown there is deficient mGluR5 function in *Tsc2*$^{+/-}$ mice, and proposed a novel method of treatment for the cognitive impairments associated with TSC by enhancing mGluR5 signaling with PAMs. Currently, the only potential therapy directed at the core disturbances in TSC is the inhibition of mTOR activity by the drug rapamycin. However, rapamycin has strong immunosuppressive properties, and therefore is not ideal for use as a chronic intervention treatment. The benefit of mGluR5 PAMs is that they does not share this detrimental property, and they target specifically the synaptic mechanisms that are likely responsible for the cognitive and behavioral impairments in TSC. Also, because mGluR5 PAMs do not directly activate or inhibit mGluR activity, but rather modulate the receptors' response to endogenous activation, they have the attribute of enhancing mGluR5 activity in a physiologically relevant way. We have demonstrated the potential of mGluR5 positive allosteric modulation as a treatment for TSC and believe they may provide the TSC community with a novel, safe and effective treatment directed at the core central nervous system disruptions inherent to the disorder.

"So what section"

This work has profound implications for treatment of autism and intellectual disability as well. TSC and FXS represent two leading genetic risk factors for autism. Although great strides have been made in identifying genetic variation that correlates with non-syndromic autism, there is little known about autism pathophysiology—knowledge that is essential for developing effective therapies. Our test of the hypothesis that the *Fmr1*$^{-/y}$ and *Tsc2*$^{+/-}$ models of FXS and TSC have a shared synaptic pathophysiology revealed instead that they are at opposite ends of a spectrum: the *Fmr1* mutation causes exaggerated synaptic protein synthesis and LTD that are corrected by inhibition of mGluR5, whereas the *Tsc2* mutation causes diminished synaptic protein synthesis and LTD that are corrected by augmentation of mGluR5 (**Fig. 7A**). Moreover, the opposing effects of these mutations balance one another at synaptic and behavioral levels in the double mutant. This finding is interesting in light of recent discoveries that gain- and loss-of-function mutations in individual genes, such as *MECP2*, can often yield syndromes with overlapping features, such as epilepsy, cognitive impairment, and autism. Our findings reveal that even genetically heterogeneous causes of autism may produce similar deficits by bidirectional deviations from normal on a common functional axis. The important implication is that therapies designed to correct one cause of autism are not likely to be effective for all other causes, and might well be deleterious. It will be critical to understand where a patient lies on the spectrum of synaptic function to choose an appropriate therapy for autism and other psychiatric disorders.

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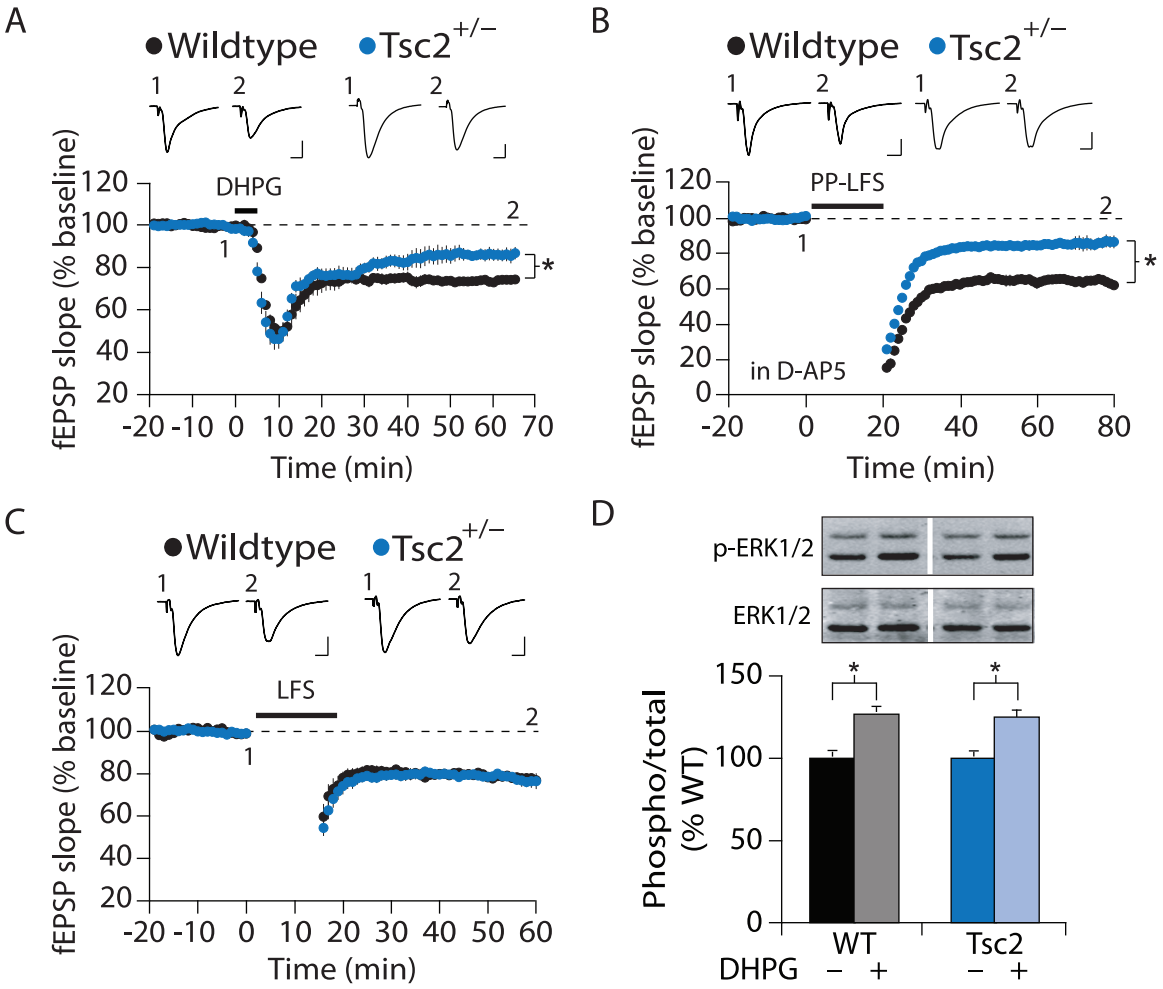
Figure 1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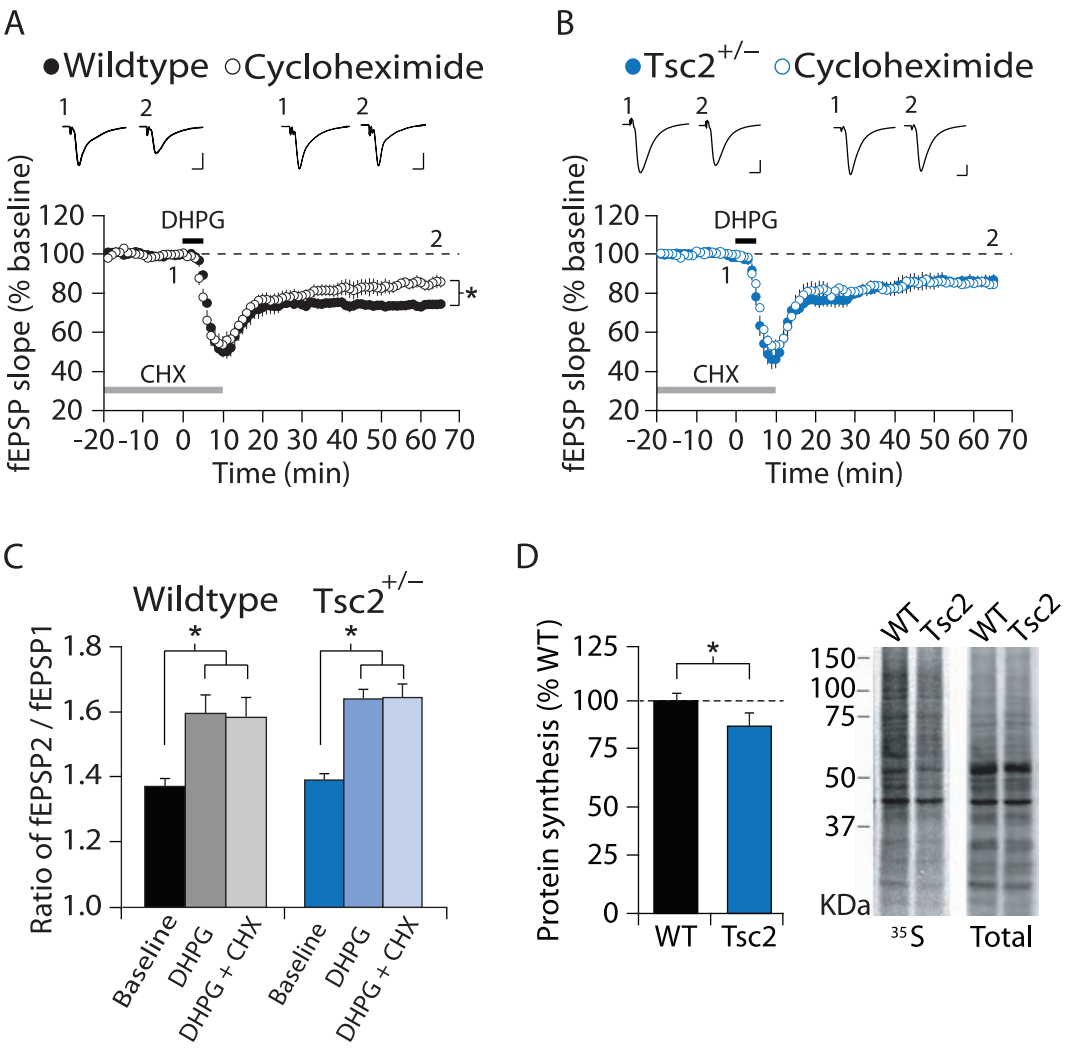
Tsc2+/–
DHPG
fEPSP slope (% baseline)
Time (min)
B
PP-LFS
in D-AP5
C
LFS
D
p-ERK1/2
ERK1/2
Phospho/total (% WT)
WT
Tsc2
DHPG

Figure 2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Cycloheximide
fEPSP slope (% baseline)
DHPG
CHX
Time (min)
B
Tsc2+/−
Cycloheximide
C
Wildtype
Tsc2+/−
Ratio of fEPSP2 / fEPSP1
Baseline
DHPG
DHPG + CHX
D
Protein synthesis (% WT)
WT
Tsc2
KDa
35S
Total

Figure 3

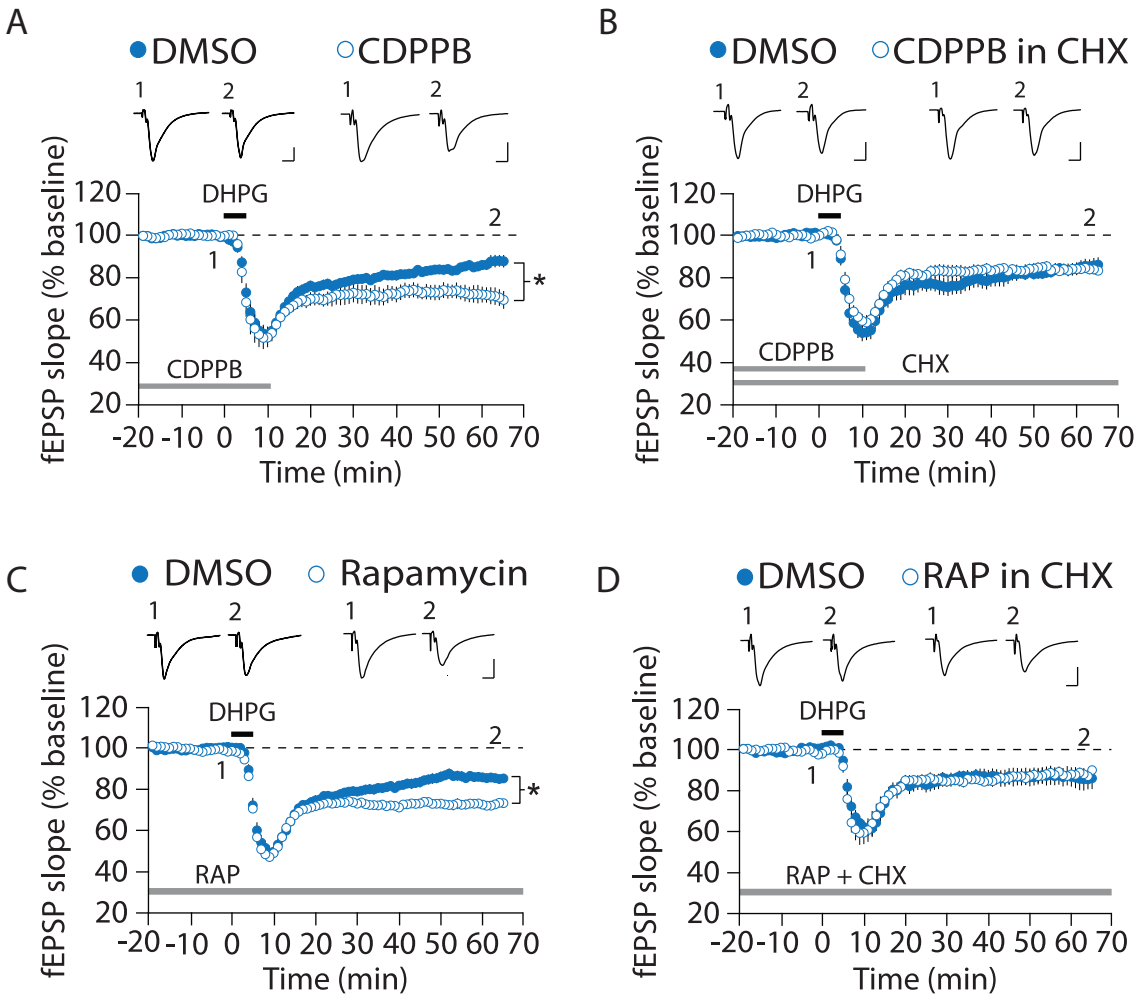
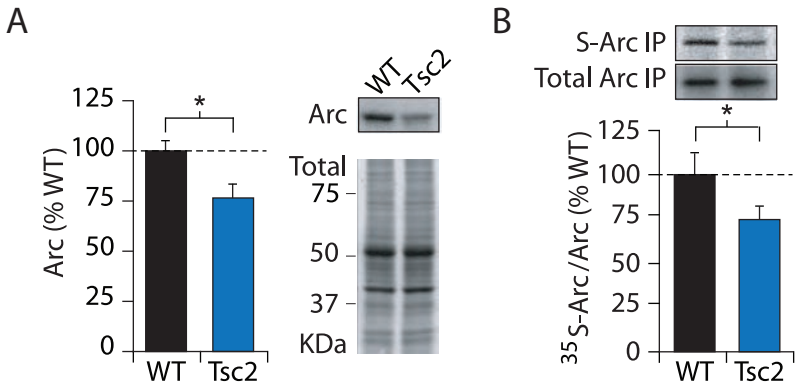

A
DMSO
CDPPB
1
2
DHPG
CDPPB
fEPSP slope (% baseline)
Time (min)
B
DMSO
CDPPB in CHX
CHX
C
DMSO
Rapamycin
RAP
D
RAP in CHX
RAP + CHX

Figure 4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100
75
50
25
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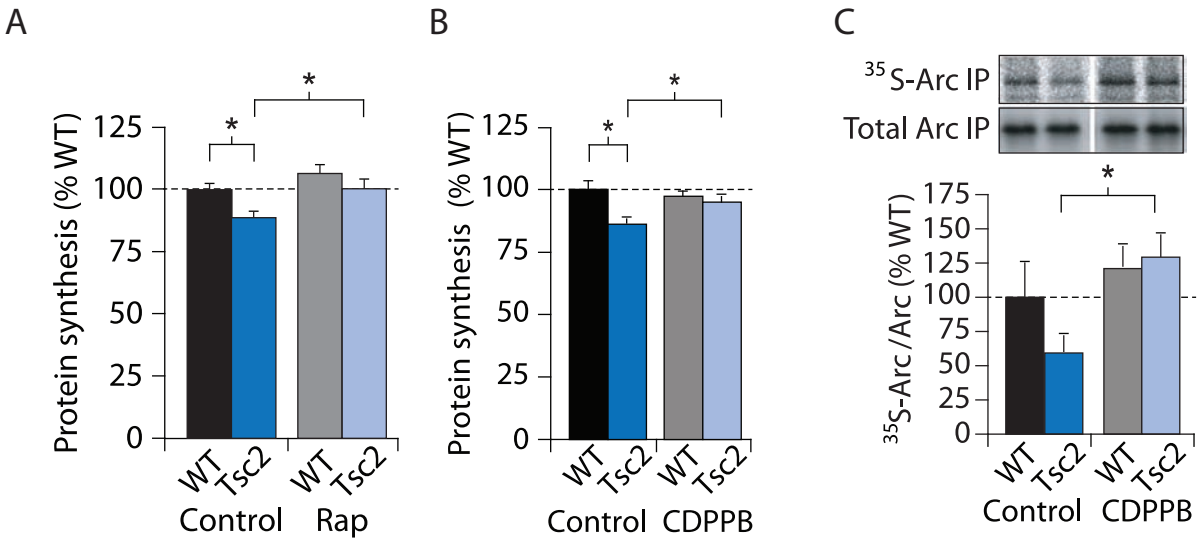
Arc (% WT)
*
WT
Tsc2
WT
Tsc2
Arc
Total
75
50
37
KDa
B
S-Arc IP
Total Arc IP
*
125
100
75
50
25
0
35S-Arc/Arc (% WT)
WT
Tsc2

Figure 5


A
B
C
Protein synthesis (% WT)
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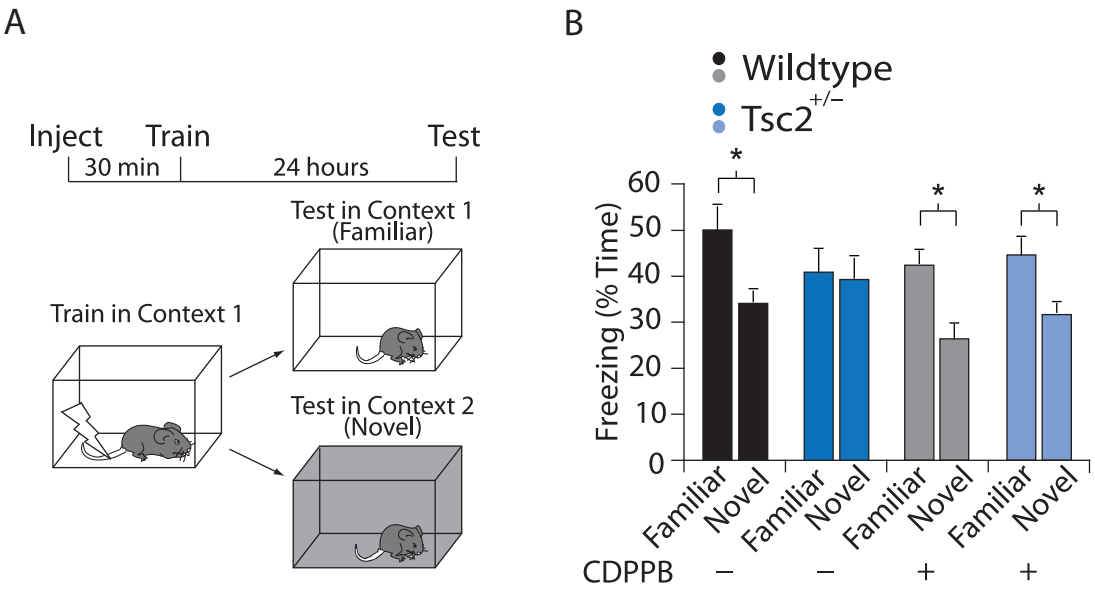
100
75
50
25
0
*
*
WT
Tsc2
WT
Tsc2
Control
Rap
Protein synthesis (% WT)
125
100
75
50
25
0
*
*
WT
Tsc2
WT
Tsc2
Control
CDPPB
35S-Arc IP
Total Arc IP
35S-Arc /Arc (% WT)
175
150
125
100
75
50
25
0
*
WT
Tsc2
WT
Tsc2
Control
CDPPB

Figure 6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Inject
Train
Test
30 min
24 hours
Test in Context 1
(Familiar)
Train in Context 1
Test in Context 2
(Novel)
B
Wildtype
Tsc2+/–
Freezing (% Time)
60
50
40
30
20
10
0
*
*
*
Familiar
Novel
Familiar
Novel
Familiar
Novel
Familiar
Novel
CDPPB
–
–
+
+

Figure 7

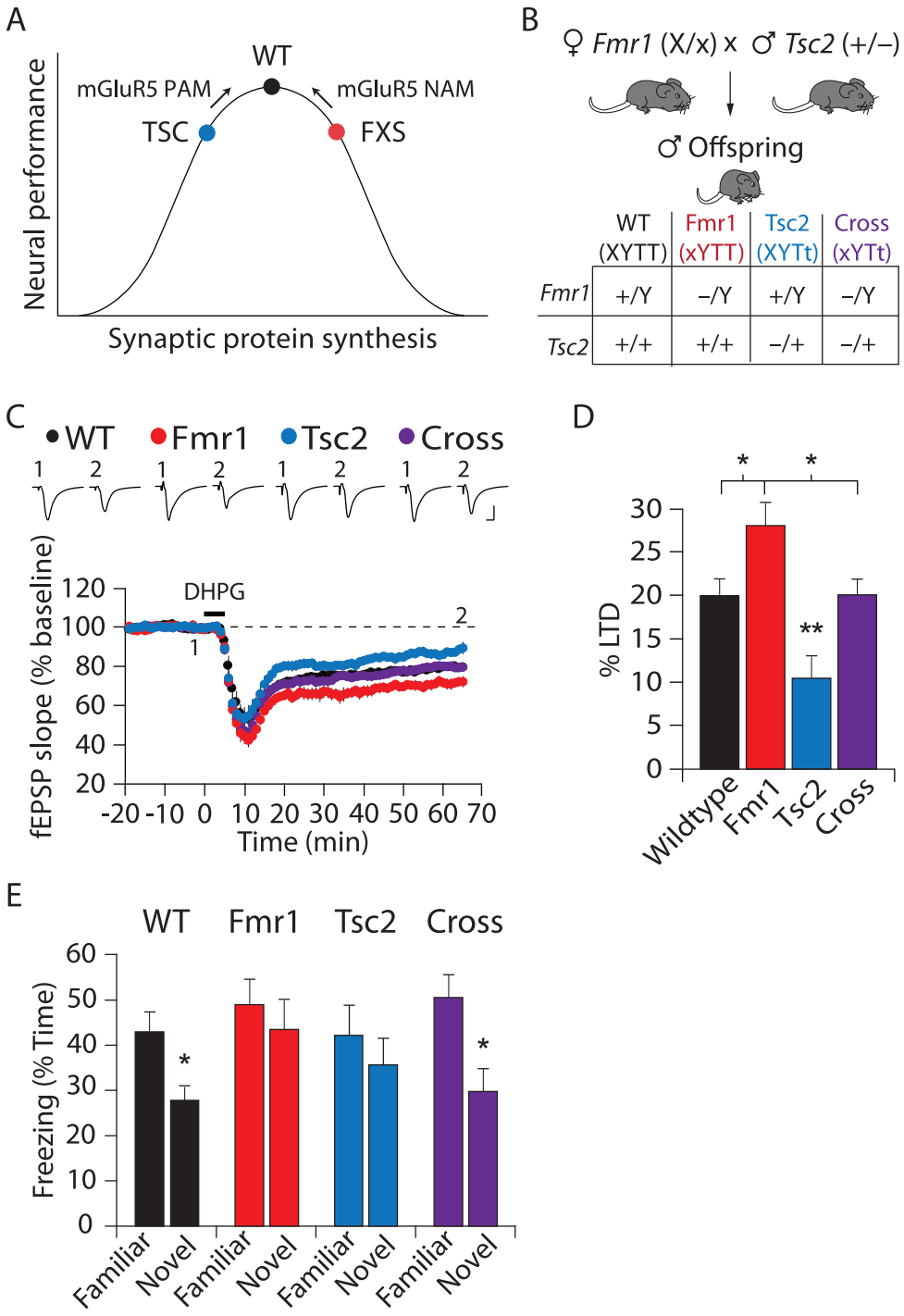
A
WT
mGluR5 PAM
mGluR5 NAM
TSC
FXS
Neural performance
Synaptic protein synthesis
B
♀ Fmr1 (X/x) x ♂ Tsc2 (+/–)
♂ Offspring
WT (XYTT)
Fmr1 (xYTT)
Tsc2 (XYTt)
Cross (xYTt)
Fmr1 +/Y –/Y +/Y –/Y
Tsc2 +/+ +/+ –/+ –/+
C
WT Fmr1 Tsc2 Cross
DHPG
fEPSP slope (% baseline)
Time (min)
D
% LTD
Wildtype
Fmr1
Tsc2
Cross
E
WT Fmr1 Tsc2 Cross
Freezing (% Time)
Familiar
Novel

Figure 1. *Tsc2*$^{+/-}$ mice have a specific deficit in mGluR-LTD

(A) DHPG induces significantly less LTD in slices from *Tsc2*$^{+/-}$ mice as compared to slices from littermate WT mice (74.3 ± 1.4%, n = 5 animals, 10 slices; *Tsc2*$^{+/-}$: 86.3 ± 3.1%, n = 6 animals, 12 slices; *p = 0.004). **(B)** Synaptically-induced mGluR-LTD, elicited by delivering pairs of pulses (50 ms interstimulus interval) at 1 Hz for 20 minutes (PP-LFS, 1200 pulses) in the presence of the NMDA receptor antagonist D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5, 50 μM), is also deficient in slices from *Tsc2*$^{+/-}$ mice (WT: 65.1 ± 2.1%, n = 3 animals, 9 slices; *Tsc2*$^{+/-}$: 85.0 ± 2.5%, n = 4 animals, 11 slices; *p = 0.003). **(C)** The magnitude of NMDA receptor-dependent LTD evoked by low frequency stimulation (LFS, 900 pulses at 1 Hz) does not differ between genotypes (WT: 79.8 ± 1.6%, n = 4 animals, 6 slices; *Tsc2*$^{+/-}$: 79.4 ± 1.9%, n = 6 animals, 6 slices; p = 0.610). **(D)** Hippocampal slices were stimulated with 50 μM DHPG for 5 min, and ERK1/2 activation (phosphorylation) assessed via immunoblot (WT: 100.0 ± 6.1%, WT DHPG: 119.6 ± 5.5%, *Tsc2*$^{+/-}$: 97.5 ± 5.6%, *Tsc2*$^{+/-}$ DHPG: 116.2 ± 3.9%; ANOVA: genotype p = 0.623, treatment *p = 0.0008, genotype x treatment p = 0.923; n = 9 animals). Results reveal that DHPG significantly increases ERK1/2 activation in both WT (*p = 0.040) and *Tsc2*$^{+/-}$ (*p = 0.003). Error bars represent SEM.

Figure 2. Protein-synthesis-dependent component of mGluR-LTD is absent in *Tsc2*$^{+/-}$ mice.

(A) LTD is significantly attenuated by pretreatment with the protein synthesis inhibitor cycloheximide (CHX, 60 μM, gray bar) in slices from WT animals (control: 74.3 ± 1.4%, n = 5 animals, 10 slices; CHX: 85.2 ± 2.8%, n = 4 animals, 7 slices; *p = 0.014). **(B)** CHX treatment has no effect on DHPG-LTD in slices from *Tsc2*$^{+/-}$ mice (control: 86.3 ± 3.1%, n = 6 animals, 12 slices; CHX: 85.3 ± 3.2%, n = 4 animals, 7 slices, p = 0.796). ANOVA: genotype *p = 0.041, treatment p = 0.089, genotype x treatment *p = 0.045. **(C)** Presynaptic LTD is not affected by genotype or CHX (see also **Fig. S1**). DHPG significantly increased PPF in slices from both WT and *Tsc2*$^{+/-}$ mice (PPF with a 50 ms inter-stimulus interval: WT baseline: 1.37 ± 0.02, WT DHPG: 1.59 ± 0.06, n = 5 animals, 9 slices, *p = 0.003; *Tsc2*$^{+/-}$ baseline: 1.39 ± 0.02, *Tsc2*$^{+/-}$ DHPG: 1.64 ± 0.03, n = 5 animals, 9 slices, *p = 0.001) and this effect was not blocked by CHX (WT DHPG + CHX: 1.58 ± 0.06, n = 7 animals, 11 slices, p = 0.89; *Tsc2*$^{+/-}$ DHPG + CHX: 1.64 ± 0.04, n = 6 animals, 7 slices, p = 0.94). **(D)** Metabolic labeling of hippocampal slices reveals a significant reduction of basal protein synthesis in *Tsc2*$^{+/-}$ mice (WT: 100.0 ± 3.1%, *Tsc2*$^{+/-}$: 88.2

± 3.3%, n = 13 animals; *p = 0.043). Differences in protein synthesis are exemplified by representative autoradiograph and total protein stain of the same membrane.

Figure 3. Deficit mGluR-LTD can be reversed by inhibition of mTOR or positive modulation of mGluR5 in *Tsc2*$^{+/-}$ mice.

(A) Pretreatment of slices with the mTORC1 inhibitor rapamycin (RAP, 20 nM, gray bar) significantly enhances DHPG-induced LTD in slices from *Tsc2*$^{+/-}$ mice (DMSO: 85.7 ± 2.1%, n = 8 animals, 17 slices; RAP: 72.9 ± 1.8%, n = 7 animals, 18 slices; *p = 0.002). **(B)** The rescue by rapamycin of DHPG-induced LTD in *Tsc2*$^{+/-}$ mice is prevented by the protein synthesis inhibitor cycloheximide (DMSO: 87.1 ± 4.7%, n = 6 animals, 10 slices; RAP: 88.1 ± 2.4%, n = 7 animals, 9 slices; p = 0.796). ANOVA: rapamycin treatment *p = 0.043, cycloheximide treatment *p = 0.004, rapamycin x cycloheximide *p = 0.018. **(C)** Pretreatment of slices from *Tsc2*$^{+/-}$ mice with CDPPB (10 μM, gray bar) significantly enhances DHPG-induced LTD (DMSO: 86.4 ± 2.5%, n = 8 animals, 13 slices; CDPPB: 71.7 ± 3.9%, n = 7 animals, 12 slices; *p < 0.001). **(D)** CDPPB treatment fails to enhance DHPG-induced LTD in *Tsc2*$^{+/-}$ mice when co-applied with the protein synthesis inhibitor cycloheximide (DMSO: 89.0 ± 4.4% n = 8 animals, 10 slices; CDPPB: 83.9 ± 2.1%, n = 7 animals, 9 slices; p = 0.64). ANOVA: CDPPB treatment *p = 0.008, CHX treatment p = 0.087, CDPPB x CHX *p = 0.034.

Figure 4. Deficient Arc translation in the hippocampus of *Tsc2*$^{+/-}$ mice.

(A) Immunoblotting experiments show that Arc expression is significantly reduced in *Tsc2*$^{+/-}$ hippocampal slices (WT: 100.0 ± 4.7%, *Tsc2*$^{+/-}$: 76.6 ± 6.4%, n = 12 animals; *p = 0.005). **(B)** Arc translation was measured by metabolic labeling of hippocampal slices, followed by immunoprecipitation of Arc. Comparison of the ratios of ^{35}S-incorporated : total Arc reveals a significant reduction in Arc translation in the *Tsc2*$^{+/-}$ hippocampus (WT: 100.0 ± 11.5%, *Tsc2*$^{+/-}$: 74.7 ± 6.8%, n = 19 animals; *p = 0.0498).

Figure 5. Rescue of protein synthesis deficit in *Tsc2*$^{+/-}$ mice by inhibiting mTOR or augmenting mGluR5 signaling

(A) Metabolic labeling experiments show that rapamycin (20 nM) normalizes protein synthesis in the *Tsc2*$^{+/-}$ hippocampus to WT levels (WT DMSO: 100.0 ± 2.5%, WT RAP: 106.5 ± 3.6%,

Tsc2$^{+/-}$ DMSO: 88.8 ± 2.6%, *Tsc2*$^{+/-}$ RAP: 100.4 ± 3.9%; ANOVA: genotype *p = 0.008, treatment *p = 0.006, genotype x treatment p = 0.430; t-test: WT vs. *Tsc2*$^{+/-}$ DMSO *p = 0.003; WT vs. *Tsc2*$^{+/-}$ RAP p = 0.344; *Tsc2*$^{+/-}$ DMSO vs. RAP *p = 0.037; n = 22 animals). Error bars represent SEM. **(B)** CDPPB (10 μM) restores protein synthesis in the *Tsc2*$^{+/-}$ hippocampus to WT levels (WT DMSO: 100.0 ± 3.2%, WT CDPPB: 97.2 ± 1.9%, *Tsc2*$^{+/-}$ DMSO: 86.1 ± 2.7%, *Tsc2*$^{+/-}$ CDPPB: 94.9 ± 3.0%; ANOVA: genotype *p = 0.006, treatment p = 0.275, genotype x treatment *p = 0.041; t-test: WT vs. *Tsc2*$^{+/-}$ DMSO *p = 0.012; WT vs. *Tsc2*$^{+/-}$ CDPPB p = 0.538; *Tsc2*$^{+/-}$ DMSO vs. CDPPB *p = 0.049; n = 17 animals). **(C)** CDPPB exposure significantly increases Arc translation in the *Tsc2*$^{+/-}$ hippocampus (WT DMSO 100.0 ± 28.2%, WT CDPPB 121.0 ± 21.2%, *Tsc2*$^{+/-}$ DMSO 59.2 ± 7.0%, *Tsc2*$^{+/-}$ CDPPB 129.4 ± 20.3%; ANOVA genotype p = 0.554, treatment *p = 0.0094, genotype x treatment p = 0.114; t-test: *Tsc2*$^{+/-}$ DMSO vs. CDPPB *p = 0.026; n = 6 animals). Error bars represent SEM.

Figure 6. mGluR5 PAM treatment rescues context discrimination deficit in *Tsc2*$^{+/-}$ mice

(A) Experimental design of context discrimination task. **(B)** WT mice display intact memory by freezing more in the familiar context than the novel context (Black bars; Familiar: 50 ± 7.7%, n = 12; Novel: 34.1 ± 3.2%, n = 14; *p = 0.003). A single injection of CDPPB (10 mg/kg, i.p.) 30 minutes prior to training has no effect on WT context discrimination (Familiar: 42.3 ± 3.7%, n = 12; Novel: 26.4 ± 3.6%, n = 12; *p = 0.005). Control *Tsc2*$^{+/-}$ mice display a significant impairment in context discrimination (Blue bars; Familiar: 40.9 ± 5.3%, n = 11; Novel: 39.3 ± 5.2%, n = 14; p = 0.501), but this deficit is corrected by a single injection of CDPPB (Familiar: 44.5 ± 4.3%, n = 11; Novel: 31.6 ± 3%, n = 12; *p = 0.034). Error bars represent SEM.

Figure 7. Genetic cross of *Tsc2*$^{+/-}$ and *Fmr1*$^{-/y}$ mice rescues synaptic and behavioral impairments present in both single mutants

(A) The data suggest that mutations in TSC and FXS cause opposing deviations in synaptic function which impairs neuronal performance and respond to opposite alterations in mGluR5 signaling[3,43]. These results raise the possibility that introducing both mutations to a mouse may normalize aspects of neural function. **(B)** Genetic rescue strategy. Heterozygous *Tsc2* male mice (*Tsc2*$^{+/-}$) were bred with heterozygous *Fmr1* females (*Fmr1* x^{+}/x^{-}) to obtain male offspring of four genotypes: wild type (*Tsc2*$^{+/+}$, *Fmr1*$^{+/y}$), *Fmr1* KO (*Tsc2*$^{+/+}$, *Fmr1*$^{-/y}$), *Tsc2* Het (*Tsc2*$^{+/-}$,

Fmr1$^{+/y}$), and Cross (*Tsc2*$^{+/-}$, *Fmr1*$^{-/y}$). **(C)** DHPG-induced LTD is significantly decreased in slices from *Tsc2*$^{+/-}$ mice (*p = 0.002) and significantly increased in slices from *Fmr1*$^{-/y}$ mice (*p = 0.017), as compared to WT slices. DHPG-LTD in slices from *Tsc2*$^{+/-}$ x *Fmr1*$^{-/y}$ mice is comparable in magnitude to WT slices (p = 0.558). (WT: 78.9 ± 2.1%, n = 7 animals, 17 slices; *Fmr1*: 71.2 ± 2.7%, n = 7 animals, 21 slices; *Tsc2*: 89.5 ± 2.6%, n = 7 animals, 15 slices; Cross: 77.4 ± 1.8%, n = 9 animals, 19). **(D)** Summary of LTD data. Bar graphs represent percent decrease from baseline in fEPSP (average of last 5 minutes of recording ± SEM); *p < 0.05, **p < 0.01. **(E)** Both mutations cause a deficit in context discrimination that is rescued in the double mutant. WT mice (Familiar: 42.9 ± 4.6%, n = 11; Novel: 27.8 ± 3.4%, n = 12; *p = 0.024) , *Fmr1*$^{-/y}$ mice (Familiar: 49.0 ± 5.6%, n = 11; Novel: 43.5 ± 6.7%, n = 12; p = 0.483), *Tsc2*$^{+/-}$ (Familiar: 42.1 ± 6.8%, n = 12; Novel: 35.6 ± 6.0%, n = 12; p = 0.395) and *Tsc2*$^{+/-}$ x *Fmr1*$^{-/y}$ mice (Familiar: 50.5 ± 5.2%, n = 11; Novel: 29.8 ± 5.2%, n = 11; *p = 0.011). Error bars represent SEM.

ARTICLE

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Mutations causing syndromic autism define an axis of synaptic pathophysiology

Benjamin D. Auerbach[1], Emily K. Osterweil[1] & Mark F. Bear[1]

Tuberous sclerosis complex and fragile X syndrome are genetic diseases characterized by intellectual disability and autism. Because both syndromes are caused by mutations in genes that regulate protein synthesis in neurons, it has been hypothesized that excessive protein synthesis is one core pathophysiological mechanism of intellectual disability and autism. Using electrophysiological and biochemical assays of neuronal protein synthesis in the hippocampus of *Tsc2*$^{+/-}$ and *Fmr1*$^{-/y}$ mice, here we show that synaptic dysfunction caused by these mutations actually falls at opposite ends of a physiological spectrum. Synaptic, biochemical and cognitive defects in these mutants are corrected by treatments that modulate metabotropic glutamate receptor 5 in opposite directions, and deficits in the mutants disappear when the mice are bred to carry both mutations. Thus, normal synaptic plasticity and cognition occur within an optimal range of metabotropic glutamate–receptor–mediated protein synthesis, and deviations in either direction can lead to shared behavioural impairments.

More than 1% of the human population has an autism spectrum disorder (ASD), and it has been estimated that over 50% of those with autism also have intellectual disability[1]. In most cases, the cause is unknown. However, genetically defined syndromes with increased prevalence of autism and intellectual disability offer an opportunity to understand the brain pathophysiology that manifests as ASD and intellectual disability, and this knowledge can suggest potential therapies. A case in point is fragile X syndrome (FXS), caused by silencing of the *FMR1* gene and loss of the protein product, FMRP. Studies of the *Fmr1* knockout (*−/y*) mouse revealed that in the absence of FMRP, protein synthesis is increased downstream of metabotropic glutamate receptor 5 (mGluR5). Diverse mutant phenotypes in fragile X animal models have been corrected by genetic or pharmacological inhibition of mGluR5, and preliminary human clinical trials using drugs that inhibit mGluR5 have shown promise[2]. Because several other syndromic forms of ASD and intellectual disability are associated with mutations of genes that regulate messenger RNA (mRNA) translation at synapses, it has been hypothesized that altered synaptic protein synthesis might contribute generally to the autistic phenotype, including intellectual disability[3]. The aim of the current study was to test the hypothesis that a mutation responsible for another genetic syndrome associated with ASD and intellectual disability—tuberous sclerosis complex (TSC)—produces abnormalities in synaptic protein synthesis and plasticity similar to fragile X. If this were the case, treatments developed for one disorder might be beneficial for the other, and possibly for autism and intellectual disability more broadly.

TSC mutations affect synaptic function

The choice of TSC was guided by several considerations. Like FXS, (1) TSC is a single-gene disorder with core symptoms of ASD and intellectual disability, (2) the affected gene(s) lie in a signalling pathway that couples cell surface receptors to mRNA translation, (3) there are well validated mouse models of the disease and (4) some mutant phenotypes in these mouse models have responded to pharmacological treatments that affect protein synthesis[4–6]. The disease is caused by heterozygous mutations in the genes encoding TSC1 or TSC2 proteins that together form the TSC1/2 complex. TSC1/2 acts to inhibit Rheb, a Ras family GTPase with high specificity for mTOR within a protein complex called mTORC1. Rheb activation of mTORC1 can stimulate mRNA translation and cell growth, and excessive mTORC1 activation is believed to be pathogenic in TSC[7]. TSC is characterized by the growth of hamartomas that are believed to result from inactivation of the functional allele within the tumour cells[8,9]. Although some neurological manifestations of TSC are thought to be related to tumour growth in the cerebral cortex, others including cognitive impairment and autism have been proposed to result from abnormal signalling at synapses[10]. Consistent with this idea, mice engineered to carry heterozygous loss-of-function mutations in *Tsc1* or *Tsc2* have been shown to have hippocampus-dependent learning and memory deficits without having tumours in the brain or seizures[4,11]. Here we chose the *Tsc2*$^{+/-}$ mouse model because *TSC2* mutations are more common and produce a more severe phenotype in humans[12], and this animal model is in widespread use[4,13–15]. Of particular significance, postnatal treatment of *Tsc2*$^{+/-}$ mice with the mTORC1 inhibitor rapamycin was previously shown to ameliorate hippocampal memory impairments, suggesting the exciting possibility that some aspects of TSC, like FXS, might be amenable to drug therapy[4].

A prominent hypothesis is that synaptic dysfunction in TSC relates to increased protein synthesis in response to elevated mTORC1 activity[16]. Signalling by mTORC1 has been suggested to contribute to the coupling of mGluR5 to protein synthesis and, although still controversial, it has been proposed that elevated mTOR activity might also be a cause of elevated protein synthesis in the *Fmr1*$^{-/y}$ mouse[17]. A sensitive electrophysiological read-out of local mRNA translation in response to mGluR5 activation is long-term synaptic depression (LTD) in area CA1 of the hippocampus[18,19]. Indeed, it was exaggerated LTD in the *Fmr1*$^{-/y}$ mouse that led to the mGluR theory of FXS[20,21]. Therefore, to test the hypothesis of a shared pathophysiology between TSC and FXS, we first examined mGluR-LTD in the hippocampus of male *Tsc2*$^{+/-}$ mice.

Protein synthesis and mGluR–LTD in *Tsc2*$^{+/-}$ mice

LTD was induced by activation of group 1 (Gp1) mGluRs (mGluR 1 and 5) with the selective agonist DHPG ((R,S)-3,5-dihydroxyphenylglycine)

[1]Howard Hughes Medical Institute, The Picower Institute for Learning and Memory, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.

in hippocampal slices[19]. Unexpectedly, we discovered that DHPG-induced LTD was deficient rather than enhanced in the hippocampus of *Tsc2*$^{+/-}$ mice, compared with wild-type (WT) controls (Fig. 1a). A similar deficit was observed when mGluR-LTD was induced by patterned electrical stimulation of Schaffer collateral synapses (Fig. 1b). In agreement with a previous report[4], basal synaptic transmission in CA1 appeared normal in the *Tsc2*$^{+/-}$ mice, indicating that the impairment in mGluR-LTD is not due to general disruption of synaptic function (Supplementary Fig. 1). Moreover, there was no difference in the magnitude of the NMDA (*N*-methyl-D-aspartate)-receptor-dependent form of LTD between WT and *Tsc2*$^{+/-}$ mice (Fig. 1c), demonstrating that the deficit is specific to mGluR-LTD, as these same synapses are able to undergo activity-induced depression by a different mechanism. To test the possibility of a general disruption in Gp 1 mGluR function, we examined DHPG-induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), a common measure of Gp1 mGluR signalling and a critical step for mGluR-mediated protein synthesis and LTD[22,23]. Basal ERK1/2 phosphorylation and DHPG-induced increases in ERK1/2 phosphorylation are unaltered in *Tsc2*$^{+/-}$ mice (Fig. 1d). These results suggest that the deficit in mGluR-LTD seen in the *Tsc2*$^{+/-}$ hippocampus is not due to a global dysregulation of synaptic function or Gp 1 mGluR signalling.

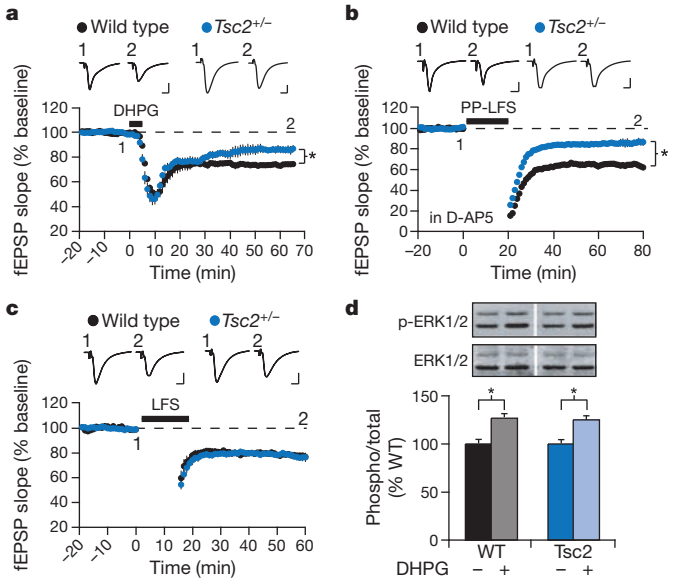

Figure 1 | *Tsc2*$^{+/-}$ mice have a specific deficit in mGluR-LTD. **a**, DHPG induces significantly less LTD in slices from *Tsc2*$^{+/-}$ mice versus littermate WT mice (WT: 74.3 ± 1.4%, $n = 5$ animals, 10 slices; *Tsc2*$^{+/-}$: 86.3 ± 3.1%, $n = 6$ animals, 12 slices; $^*P = 0.004$). In this and all subsequent electrophysiology figures, representative field potential traces (average of 10 sweeps) were taken at times indicated by numerals and scale bars equal to 0.5 mV, 5 ms, unless stated otherwise. Error bars, s.e.m. **b**, Synaptically induced mGluR-LTD, elicited by delivering pairs of pulses (50 ms inter-stimulus interval) at 1 Hz for 20 min (paired-pulse low-frequency synaptic stimulation (PP-LFS), 1,200 pulses) in the presence of the NMDA receptor antagonist D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5, 50 μM), is also deficient in slices from *Tsc2*$^{+/-}$ mice (WT: 65.1 ± 2.1%, $n = 3$ animals, 9 slices; *Tsc2*$^{+/-}$: 85.0 ± 2.5%, $n = 4$ animals, 11 slices; $^*P = 0.003$). **c**, The magnitude of NMDA receptor-dependent LTD evoked by low-frequency stimulation (LFS, 900 pulses at 1 Hz) does not differ between genotypes (WT: 79.8 ± 1.6%, $n = 4$ animals, 6 slices; *Tsc2*$^{+/-}$: 79.4 ± 1.9%, $n = 6$ animals, 6 slices; $P = 0.610$). **d**, Hippocampal slices were stimulated with 50 μM DHPG for 5 min, and ERK1/2 activation (phosphorylation) assessed by immunoblot (normalized WT: 100.0 ± 6.1%; WT DHPG: 119.6 ± 5.5%; *Tsc2*$^{+/-}$: 97.5 ± 5.6%; *Tsc2*$^{+/-}$ DHPG: 116.2 ± 3.9%; ANOVA: genotype $P = 0.623$, treatment $^*P = 0.0008$, genotype × treatment $P = 0.923$; $n = 9$ animals). Results reveal that DHPG significantly increases ERK1/2 activation in both WT ($^*P = 0.040$) and *Tsc2*$^{+/-}$ ($^*P = 0.003$). Error bars, s.e.m.

mGluR-LTD in area CA1 of the hippocampus is expressed by two independent mechanisms: reduced probability of presynaptic glutamate release[24–26] and reduced expression of postsynaptic AMPA receptors[25,27]. In WT animals, the postsynaptic modification is known to require immediate translation of mRNAs available in the dendrites of hippocampal pyramidal neurons[18,28]. Accordingly, we found that LTD in WT mice at the age range examined (postnatal day (P) 25–35) is reliably reduced by the protein synthesis inhibitor cycloheximide (60 μM; Fig. 2a). The presynaptic component of LTD was monitored by measuring paired-pulse facilitation, which showed a persistent increase following DHPG that reflects reduced probability of glutamate released at the presynaptic terminal[24–26]. Changes in paired-pulse facilitation were not inhibited by cycloheximide (Supplementary Fig. 2 and Fig. 2c), suggesting that residual LTD in the presence of the drug is expressed presynaptically. Although LTD was reduced in *Tsc2*$^{+/-}$ mice, the persistent paired-pulse facilitation change after DHPG was no different than in WT, suggesting a deficient postsynaptic modification (Supplementary Fig. 2 and Fig. 2c). Indeed, unlike WT, cycloheximide treatment had no effect on LTD in the *Tsc2*$^{+/-}$ animals (Fig. 2b). These data suggest a selective loss of the protein-synthesis-dependent component of LTD in the mutant mice.

These electrophysiological results in the *Tsc2*$^{+/-}$ hippocampus stand in stark contrast to the *Fmr1*$^{-/y}$ mouse in which mGluR-LTD is exaggerated[20]. In the fragile X mouse model, increased LTD correlates with an increased rate of basal mRNA translation downstream of mGluR5. Therefore we were compelled to examine protein synthesis in hippocampal slices from the *Tsc2*$^{+/-}$ mouse as previously described for the *Fmr1*$^{-/y}$ mouse[23]. Consistent with the mGluR-LTD findings, we found a small but significant decrease in [^{35}S]methionine/cysteine incorporation into protein under basal conditions in the hippocampus of *Tsc2*$^{+/-}$ mice (Fig. 2d). This finding suggested the possibility that protein(s) required for mGluR-LTD are deficiently translated in the hippocampus of *Tsc2*$^{+/-}$ mice. To test this idea we examined levels of Arc, a plasticity-related protein that is rapidly synthesized in response to Gp 1 mGluR activation and is required for mGluR-LTD[29,30]. Interestingly, we found that Arc expression is decreased in *Tsc2*$^{+/-}$ hippocampal slices (Fig. 2e). To determine whether this decrease was due to diminished translation, we measured the amount of newly synthesized Arc in *Tsc2*$^{+/-}$ slices by performing immunoprecipitation experiments on metabolically labelled slices (see Methods)[23]. Examination of the ^{35}S-incorporated fraction revealed a significant reduction in Arc translation in the hippocampus of *Tsc2*$^{+/-}$ mice (Fig. 2f). Control immunoprecipitations using non-immune IgG confirmed that our measurements were specific for Arc (Supplementary Fig. 4). These results suggest that mGluR-LTD is deficient in the *Tsc2*$^{+/-}$ hippocampus because of a decrease in the translation of the proteins required to stabilize LTD, including Arc.

LTD deficit caused by excess mTOR activity

As in the human disease, the germline mutation in *Tsc2* can have myriad secondary consequences on neural development that could contribute to the observed LTD and protein synthesis phenotypes. To test the hypothesis that the deficient mGluR-LTD seen in *Tsc2*$^{+/-}$ mice is a specific consequence of unregulated mTOR activity, we examined the effects of the mTORC1 inhibitor rapamycin. We found that acute rapamycin treatment (20 nM) restored mGluR-LTD in the *Tsc2*$^{+/-}$ mice to WT levels (Fig. 2g), whereas this same treatment had no effect on mGluR-LTD in slices from WT mice (Supplementary Fig. 3). This rescue is due specifically to the recovery of the protein-synthesis-dependent component of LTD, as the effect of rapamycin in *Tsc2*$^{+/-}$ mice was eliminated in the presence of cycloheximide (Fig. 2h). The same rapamycin treatment also restored basal protein synthesis rates in *Tsc2*$^{+/-}$ hippocampal slices back to WT levels (Fig. 2i). The simple model that best fits the data is that unregulated mTOR activity caused by the *Tsc2*$^{+/-}$ mutation suppresses the protein synthesis that is required for mGluR-LTD (Fig. 3a).

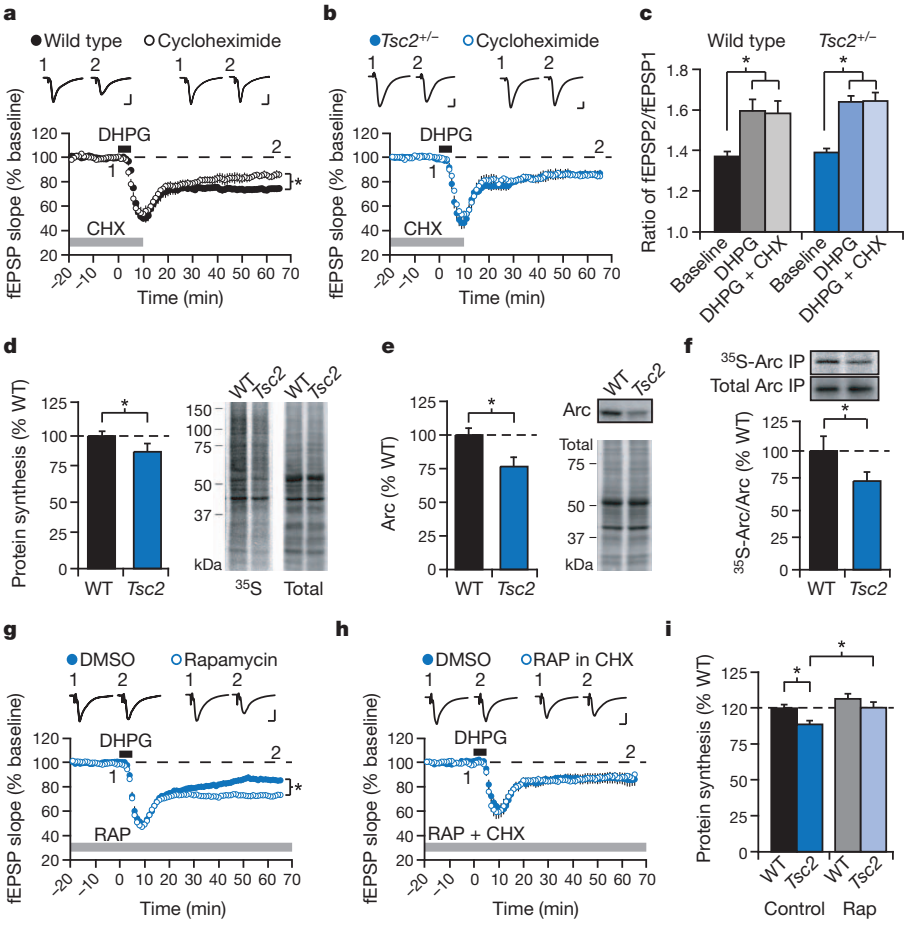

Figure 2 | Excessive mTOR activity suppresses the protein-synthesis-dependent component of mGluR-LTD. **a**, LTD is significantly attenuated by pretreatment with the protein synthesis inhibitor cycloheximide (CHX, 60 μM, grey bar) in slices from WT animals (control: 74.3 ± 1.4%, $n = 5$ animals, 10 slices; CHX: 85.2 ± 2.8%, $n = 4$ animals, 7 slices; $^*P = 0.014$). **b**, CHX treatment has no effect on LTD in slices from $Tsc2^{+/-}$ mice (control: 86.3 ± 3.1%, $n = 6$ animals, 12 slices; CHX: 85.3 ± 3.2%, $n = 4$ animals, 7 slices, $P = 0.796$). ANOVA: genotype $^*P = 0.041$, treatment $P = 0.089$, genotype × treatment $^*P = 0.045$. **c**, Presynaptic LTD is not affected by genotype or CHX (see also Supplementary Fig. 2). DHPG significantly increased paired-pulse facilitation in slices from both WT and $Tsc2^{+/-}$ mice (paired-pulse facilitation with a 50 ms inter-stimulus interval: WT baseline: 1.37 ± 0.02, WT DHPG: 1.59 ± 0.06, $n = 5$ animals, 9 slices, $^*P = 0.003$; $Tsc2^{+/-}$ baseline: 1.39 ± 0.02, $Tsc2^{+/-}$ DHPG: 1.64 ± 0.03, $n = 5$ animals, 9 slices, $^*P = 0.001$) and this effect was not blocked by CHX (WT DHPG + CHX: 1.58 ± 0.06, $n = 7$ animals, 11 slices, $P = 0.89$; $Tsc2^{+/-}$ DHPG + CHX: 1.64 ± 0.04, $n = 6$ animals, 7 slices, $P = 0.94$). **d**, Metabolic labelling of hippocampal slices reveals a significant reduction of basal protein synthesis in $Tsc2^{+/-}$ mice (WT: 100.0 ± 3.1%, $Tsc2^{+/-}$: 88.2 ± 3.3%, $n = 13$ animals; $^*P = 0.043$). Differences in protein synthesis are exemplified by representative autoradiograph and total protein stain of the same membrane. **e**, Immunoblotting experiments show that Arc expression is significantly reduced in $Tsc2^{+/-}$ hippocampal slices (WT: 100.0 ± 4.7%, $Tsc2^{+/-}$: 76.6 ± 6.4%, $n = 12$ animals; $^*P = 0.005$). **f**, Arc translation was measured by metabolic labelling of hippocampal slices, followed by immunoprecipitation of Arc. Comparison of the ratios of ^{35}S-incorporated-to-total Arc reveals a significant reduction in Arc translation in the $Tsc2^{+/-}$ hippocampus (WT: 100.0 ± 11.5%, $Tsc2^{+/-}$: 74.7 ± 6.8%, $n = 19$ animals; $^*P = 0.049$). **g**, Pretreatment of slices with the mTORC1 inhibitor rapamycin (RAP, 20 nM, grey bar) significantly enhances DHPG-induced LTD in slices from $Tsc2^{+/-}$ mice (dimethylsulphoxide (DMSO): 85.7 ± 2.1%, $n = 8$ animals, 17 slices; RAP: 72.9 ± 1.8%, $n = 7$ animals, 18 slices; $^*P = 0.002$). **h**, The rescue by rapamycin of DHPG-induced LTD in $Tsc2^{+/-}$ mice is prevented by the protein synthesis inhibitor cycloheximide (DMSO: 87.1 ± 4.7%, $n = 6$ animals, 10 slices; RAP: 88.1 ± 2.4%, $n = 7$ animals, 9 slices; $P = 0.796$). ANOVA: rapamycin treatment $^*P = 0.043$, cycloheximide treatment $^*P = 0.004$, rapamycin × cycloheximide $^*P = 0.018$. **i**, Metabolic labelling experiments show that rapamycin (20 nM) normalizes protein synthesis in the $Tsc2^{+/-}$ hippocampus to WT levels (WT DMSO: 100.0 ± 2.5%, WT RAP: 106.5 ± 3.6%, $Tsc2^{+/-}$ DMSO: 88.8 ± 2.6%, $Tsc2^{+/-}$ RAP: 100.4 ± 3.9%; ANOVA: genotype $^*P = 0.008$, treatment $^*P = 0.006$, genotype × treatment $P = 0.430$; *t*-test: WT compared with $Tsc2^{+/-}$ DMSO $^*P = 0.003$; WT compared with $Tsc2^{+/-}$ RAP $P = 0.344$; $Tsc2^{+/-}$ DMSO compared with RAP $^*P = 0.037$; $n = 22$ animals). Error bars, s.e.m.

Effect of mGluR5-positive allosteric modulation

In the $Fmr1^{-/y}$ model of FXS, excessive mGluR-LTD and hippocampal protein synthesis can be corrected by reducing signalling by mGluR5[23,31]. We therefore wondered if the opposite approach of potentiating mGluR5 signalling with a positive allosteric modulator (PAM) could be beneficial in this model of TSC (Fig. 3a). PAMs are compounds that do not activate mGluR5 directly but act on an allosteric site to potentiate physiological activation of the receptor[32]. Indeed, we found that pretreatment of hippocampal slices with the mGluR5 PAM 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB[33]) restored the magnitude of mGluR-LTD in $Tsc2^{+/-}$ mice to WT levels (Fig. 3b). The rescue of LTD appears to be due specifically to recovery of the protein-synthesis-dependent component because the effect of CDPPB was completely eliminated by cycloheximide (Fig. 3c). Consistent with this conclusion, CDPPB treatment also restored basal protein synthesis levels (Fig. 3d) and rescued the deficit in Arc synthesis in the $Tsc2^{+/-}$ mice (Fig. 3e). Thus, allosteric augmentation of mGluR5 signalling can overcome the inhibitory effect of unregulated mTOR activity on the synaptic protein synthesis that supports LTD.

In an important recent study, cognitive impairments in the $Tsc2^{+/-}$ mice were shown to be significantly improved by treating the animals with the mTORC1 inhibitor rapamycin[4]. In light of our

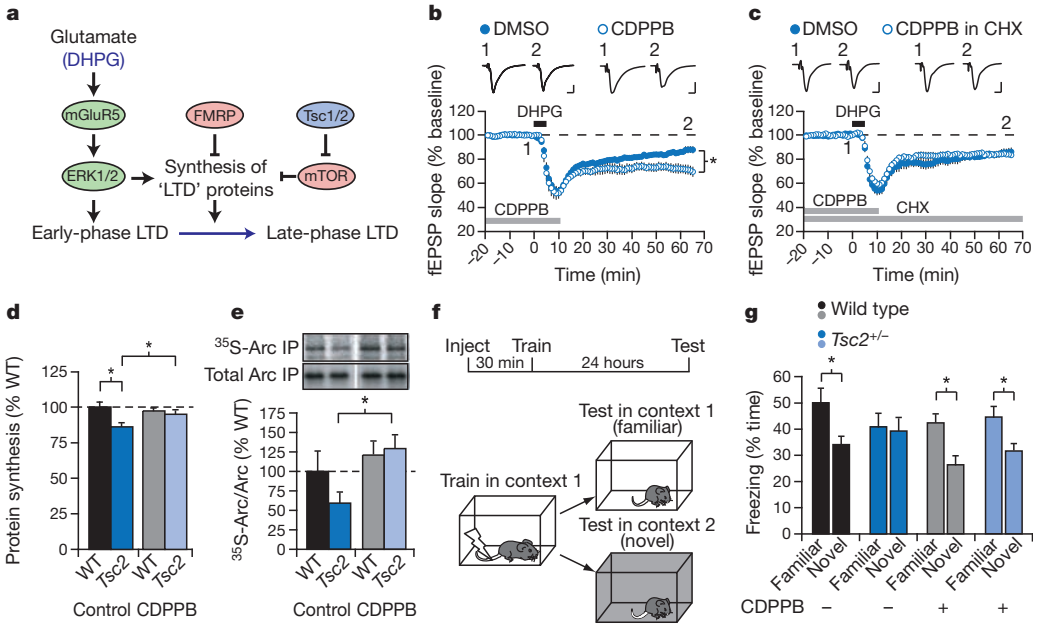

Figure 3 | Positive modulation of mGluR5 reverses synaptic and behavioural deficits in *Tsc2*$^{+/-}$ mice. **a**, Model to account for effects of *Tsc2*$^{+/-}$ and *Fmr1*$^{-/y}$ mutations on mGluR5- and protein-synthesis-dependent LTD. This model predicts that the impairment in *Tsc2*$^{+/-}$ mice can be overcome either by inhibiting mTOR with rapamycin or by augmenting mGluR5 signalling with an mGluR5 PAM. **b**, Consistent with the model, pretreatment of slices from *Tsc2*$^{+/-}$ mice with CDPPB (10 μM, grey bar) significantly enhances DHPG-induced LTD (DMSO: 86.4 ± 2.5%, $n = 8$ animals, 13 slices; CDPPB: 71.7 ± 3.9%, $n = 7$ animals, 12 slices; $^{*}P < 0.001$). **c**, CDPPB treatment fails to enhance DHPG-induced LTD in *Tsc2*$^{+/-}$ mice when co-applied with the protein synthesis inhibitor cycloheximide (DMSO: 89.0 ± 4.4%, $n = 8$ animals, 10 slices; CDPPB: 83.9 ± 2.1%, $n = 7$ animals, 9 slices; $P = 0.64$). ANOVA: CDPPB treatment $^{*}P = 0.008$, CHX treatment $P = 0.087$, CDPPB × CHX $^{*}P = 0.034$. **d**, CDPPB (10 μM) restores protein synthesis in the *Tsc2*$^{+/-}$ hippocampus to WT levels (WT DMSO: 100.0 ± 3.2%, WT CDPPB: 97.2 ± 1.9%, *Tsc2*$^{+/-}$ DMSO: 86.1 ± 2.7%, *Tsc2*$^{+/-}$ CDPPB: 94.9 ± 3.0%; ANOVA: genotype $^{*}P = 0.006$, treatment $P = 0.275$, genotype × treatment $^{*}P = 0.041$; *t*-test: WT compared with *Tsc2*$^{+/-}$ DMSO $^{*}P = 0.012$; WT compared with *Tsc2*$^{+/-}$ CDPPB $P = 0.538$; *Tsc2*$^{+/-}$ DMSO compared with CDPPB $^{*}P = 0.049$; $n = 17$ animals). **e**, CDPPB exposure significantly increases Arc translation in the *Tsc2*$^{+/-}$ hippocampus (WT DMSO 100.0 ± 28.2%, WT CDPPB 121.0 ± 21.2%, *Tsc2*$^{+/-}$ DMSO 59.2 ± 7.0%, *Tsc2*$^{+/-}$ CDPPB 129.4 ± 20.3%; ANOVA genotype $P = 0.554$, treatment $^{*}P = 0.009$, genotype × treatment $P = 0.114$; *t*-test: *Tsc2*$^{+/-}$ DMSO compared with CDPPB $^{*}P = 0.026$; $n = 6$ animals). Error bars, s.e.m. **f**, Experimental design of context discrimination task. **g**, WT mice display intact memory by freezing more in the familiar context than the novel context (black bars; familiar: 50 ± 7.7%, $n = 12$; novel: 34.1 ± 3.2%, $n = 14$; $^{*}P = 0.003$). A single injection of CDPPB (10 mg kg^{-1}, intraperitoneal) 30 min before training has no effect on WT context discrimination (familiar: 42.3 ± 3.7%, $n = 12$; novel: 26.4 ± 3.6%, $n = 12$; $^{*}P = 0.005$). Control *Tsc2*$^{+/-}$ mice display an impairment in context discrimination (blue bars; familiar: 40.9 ± 5.3%, $n = 11$; novel: 39.3 ± 5.2%, $n = 14$; $P = 0.501$), but this deficit is corrected by a single injection of CDPPB (familiar: 44.5 ± 4.3%, $n = 11$; novel: 31.6 ± 3%, $n = 12$; $^{*}P = 0.034$). Error bars, s.e.m.

electrophysiological and biochemical findings, we wondered if a similar amelioration would be observed with the mGluR5 PAM. A robust phenotype was reported to be an impairment in the ability of the *Tsc2*$^{+/-}$ mice to distinguish between familiar and novel contexts in a fear conditioning task. Advantages of this task are that the learning occurs in one trial, making it amenable to acute drug treatment, and the memory is hippocampus dependent[34]. Although a requirement for CA1 LTD per se has not been established, contextual fear discrimination does depend on both mGluR5[35] and new protein synthesis at the time of training[36]. In this assay, mice are first exposed to a distinctive context in which they receive an aversive foot shock. The next day, context discrimination is tested by dividing the animals into two groups: one is placed in the familiar context associated with the shock; the other is placed in a novel context (Fig. 3f). Context discrimination is assessed by measuring the time the animals express fear by freezing in each context. Although the WT mice clearly discriminate between contexts, the *Tsc2*$^{+/-}$ mice do not[4] (Fig. 3g). To test the effect of augmenting mGluR5 signalling, mice from both genotypes were injected intraperitoneally with CDPPB (10 mg kg^{-1}) 30 min before training. Although this treatment had no effect in the WT mice, it was sufficient to correct the deficit in context discrimination observed in the *Tsc2*$^{+/-}$ mice. These results show that augmentation of mGluR5 signalling is beneficial at the behavioural level in *Tsc2*$^{+/-}$ mice and that disrupted mGluR5 function may be relevant to cognitive impairments associated with TSC.

Fmr$^{-/y}$ and *Tsc2*$^{+/-}$ mutations cancel each other

Contrary to our initial hypothesis, we found that mutations causing FXS and TSC, two disorders associated with autism and intellectual disability, show mirror symmetrical alterations in protein-synthesis-dependent LTD and have beneficial responses to treatments that modulate mGluR5 in opposite directions (Fig. 4a). These findings raised the intriguing possibility that these two mutations could cancel one another on this functional axis. To test this hypothesis, we introduced an *Fmr1* deletion into the *Tsc2*$^{+/-}$ background by crossing *Tsc2*$^{+/-}$ males with *Fmr1*$^{+/-}$ females (Fig. 4b). This approach also enabled us to compare directly with WT the effects of the *Tsc2*$^{+/-}$ and *Fmr1*$^{-/y}$ mutations in littermates reared under identical conditions. As expected, mGluR-LTD was diminished in *Tsc2*$^{+/-}$ mice and excessive in the *Fmr1*$^{-/y}$ mice, compared with WT (Fig. 4c,d). However, mice harbouring both mutations showed mGluR-LTD that was indistinguishable from WT (Fig. 4c,d).

Although *Tsc2*$^{+/-}$ and *Fmr1*$^{-/y}$ mutations cause opposite alterations in mGluR-LTD and protein synthesis, the human disorders they are associated with have similar neurological and cognitive phenotypes. Might opposite deviations in synaptic function lead to shared cognitive impairments? To examine this question, we compared context discrimination in the *Tsc2*$^{+/-}$ and *Fmr1*$^{-/y}$ mice and discovered that indeed they do share a deficit in this measure of memory (Fig. 4e). Remarkably, instead of being exacerbated, this memory deficit was erased in the double mutants (Fig. 4e). These results suggest that the

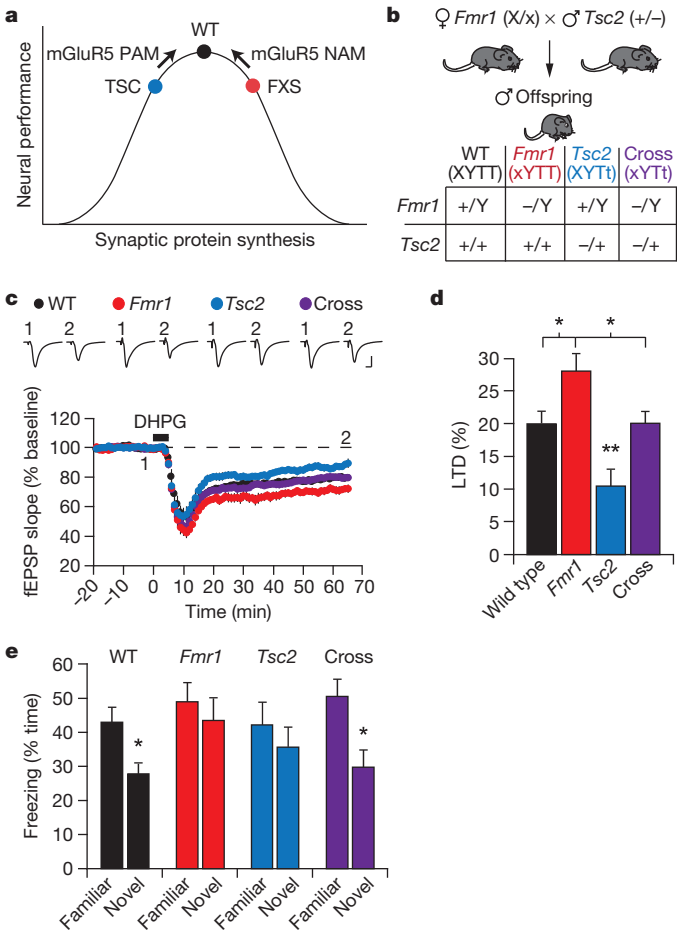

Figure 4 | Genetic cross of *Tsc2*$^{+/-}$ and *Fmr1*$^{-/y}$ mice rescues synaptic and behavioural impairments present in both single mutants. a, The data suggest that optimal synaptic function requires a narrow and tightly regulated level of synaptic protein synthesis and that deviations in either direction can impair function[3,43]. TSC and FXS fall on different ends of this spectrum and respond to opposite alterations of mGluR5 signalling. These results raise the possibility that introducing both mutations to a mouse may normalize aspects of neural function. **b**, Genetic rescue strategy. Heterozygous *Tsc2* male mice (*Tsc2*$^{+/-}$) were bred with heterozygous *Fmr1* females (*Fmr1* x^{+}/x^{-}) to obtain male offspring of four genotypes: WT (*Tsc2*$^{+/+}$, *Fmr1*$^{+/y}$), *Fmr1* KO (*Tsc2*$^{+/+}$, *Fmr1*$^{-/y}$), *Tsc2* Het (*Tsc2*$^{+/-}$, *Fmr1*$^{+/y}$) and cross (*Tsc2*$^{+/-}$, *Fmr1*$^{-/y}$). **c**, DHPG-induced LTD is significantly decreased in slices from *Tsc2*$^{+/-}$ mice (*$P = 0.002$) and significantly increased in slices from *Fmr1*$^{-/y}$ mice (*$P = 0.017$), compared with WT slices. DHPG-LTD in slices from *Tsc2*$^{+/-}$ × *Fmr1*$^{-/y}$ mice is comparable in magnitude to WT slices ($P = 0.558$). (WT: 78.9 ± 2.1%, $n = 7$ animals, 17 slices; *Fmr1*: 71.2 ± 2.7%, $n = 7$ animals, 21 slices; *Tsc2*: 89.5 ± 2.6%, $n = 7$ animals, 15 slices; cross: 77.4 ± 1.8%, $n = 9$ animals, 19 slices). **d**, Summary of LTD data. Bar graphs, percentage decrease from baseline in fEPSP (average of last 5 min of recording ± s.e.m.); *$P < 0.05$, **$P < 0.01$. **e**, Both mutations cause a deficit in context discrimination that is rescued in the double mutant. WT mice (familiar: 42.9 ± 4.6%, $n = 11$; novel: 27.8 ± 3.4%, $n = 12$; *$P = 0.024$), *Fmr1*$^{-/y}$ mice (familiar: 49.0 ± 5.6%, $n = 11$; novel: 43.5 ± 6.7%, $n = 12$; $P = 0.483$), *Tsc2*$^{+/-}$ mice (familiar: 42.1 ± 6.8%, $n = 12$; novel: 35.6 ± 6.0%, $n = 12$; $P = 0.395$) and *Tsc2*$^{+/-}$ × *Fmr1*$^{-/y}$ mice (familiar: 50.5 ± 5.2%, $n = 11$; novel: 29.8 ± 5.2%, $n = 11$; *$P = 0.011$). Error bars, s.e.m.

opposing synaptic deviations seen in *Tsc2*$^{+/-}$ and *Fmr1*$^{-/y}$ mice may manifest similarly at the behavioural level, as introducing both mutations not only reverses the disruptions of synaptic plasticity but rescues this memory impairment as well.

Discussion

LTD and protein synthesis downstream of mGluR5 have attracted attention in the context of several diseases, most notably FXS[27]. Fragile X is caused by the loss of FMRP, an mRNA-binding protein that negatively regulates translation[37,38]. In the *Fmr1*$^{-/y}$ mouse model, basal protein synthesis is elevated and LTD is exaggerated downstream of an mGluR5 signalling pathway involving ERK1/2 (ref. 23). Partial inhibition of mGluR5 corrects multiple aspects of fragile X in animal models[2,39]. Recent data suggest that the mTOR signalling pathway is also constitutively overactive in the *Fmr1*$^{-/y}$ mouse[17], but the relevance to exaggerated protein synthesis and altered synaptic function has been unclear. The current findings show that increased synaptic mTOR activity actually suppresses the protein synthesis required for LTD in the *Tsc2*$^{+/-}$ mice. The idea that reduced protein synthesis is a causative factor in the observed deficit in synaptic plasticity is supported by the finding that pharmacological rescue with both rapamycin and CDPPB is abolished by cycloheximide, and the observation that Arc is deficiently translated in the *Tsc2*$^{+/-}$ mice. There is good evidence that Arc is one of the proteins that normally must be synthesized to support mGluR5-dependent forms of long-term plasticity[29,30]. Precisely how excess mTOR activity suppresses synthesis of these plasticity proteins remains to be investigated, but possibilities include hyperphosphorylation of FMRP[40] or increased translation of a competing pool of less abundant mRNAs unrelated to LTD[7,21,30]. The fact that mGluR-LTD is altered in opposite directions in *Tsc2*$^{+/-}$ and *Fmr1*$^{-/y}$ mice, and that both deviations are corrected in the double mutants, suggests that the pool comprising LTD proteins is differentially regulated by FMRP and TSC1/2 (Fig. 3a).

The current findings also suggest a new treatment for behavioural deficits associated with TSC. Previous studies in the *Tsc2*$^{+/-}$ mouse raised the exciting possibility that cognitive aspects of the disorder might be ameliorated with rapamycin, even when treatment is begun in adulthood[4]. Our data show that an mGluR5 PAM may be similarly effective. Although rapamycin has been used clinically, it is problematic for chronic treatment because of its strong immunosuppressive properties. The benefit of mGluR5 PAMs is that they target specifically the synaptic mechanisms that are probably responsible for the cognitive and behavioural impairments in TSC.

TSC and FXS represent two leading genetic risk factors for ASD and intellectual disability[41]. Although great strides have been made in identifying genetic variation that correlates with non-syndromic autism, little is known about ASD pathophysiology, knowledge that is essential for developing effective therapies. Our test of the hypothesis that the *Fmr1*$^{-/y}$ and *Tsc2*$^{+/-}$ models of FXS and TSC have a shared synaptic pathophysiology revealed instead that they are at opposite ends of a spectrum: the *Fmr1* mutation causes exaggerated synaptic protein synthesis and LTD that are corrected by inhibition of mGluR5 (ref. 31), whereas the *Tsc2* mutation causes diminished synaptic protein synthesis and LTD that are corrected by augmentation of mGluR5 (Fig. 4a). Moreover, the opposing effects of these mutations balance one another at synaptic and behavioural levels in the double mutant. This finding is interesting in light of recent discoveries that gain- and loss-of-function mutations in individual genes, such as *MECP2*, can often yield syndromes with overlapping features, such as epilepsy, cognitive impairment and ASD[42]. Our findings reveal that even genetically heterogeneous causes of ASD and intellectual disability may produce similar deficits by bidirectional deviations from normal on a common functional axis. The important implication is that therapies designed to correct one cause of ASD are not likely to be effective for all other causes, and might well be deleterious. It will be critical to understand where a patient lies on the spectrum of synaptic function to choose an appropriate therapy for ASD and other psychiatric disorders.

METHODS SUMMARY

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Massachusetts Institute of Technology. Age-matched, male littermate mice bred on the C57Bl/6J clonal background were used in this study.

Acute hippocampal slices were prepared from P25-35 mice as previously described[31] and field excitatory postsynaptic potentials (fEPSPs) evoked by stimulation of the Schaffer collaterals were recorded in CA1 stratum radiatum with extracellular electrodes. LTD was induced by applying R,S-DHPG (50 μM) or S-DHPG (25 μM) for 5 min, or by paired-pulse low-frequency synaptic stimulation[18,19] for 20 min. Metabolic labelling, immunoblotting and immunoprecipitation experiments were performed on yoked WT and $Tsc2^{+/-}$ mice as described previously[23,43]. Context discrimination fear conditioning was performed as described previously[4]. For *in vivo* mGluR5 PAM experiments, animals received a single injection of CDPPB (10 mg kg^{-1}, intraperitoneal) 30 min before the training session. For all data sets, outliers more than 2σ from the mean were removed, and significance between more than two groups was determined using two-way analysis of variance (ANOVA) and *post hoc* Student's *t*-tests. Statistics were performed using each animal as an '*n*', with each animal represented by the mean of one to four slices for electrophysiology experiments, one slice per animal for biochemistry experiments and five to eight slices per animal for immunoprecipitation experiments. All experiments were performed blind to genotype and include interleaved controls for genotype and treatment. For detailed methods, see Supplementary Information.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Animals. *Tsc2*$^{+/-}$ male and female mutant mice on the C57Bl/6J clonal background were bred with C57Bl/6J WT partners to produce the WT and *Tsc2*$^{+/-}$ male offspring used in this study. For genetic rescue experiments, heterozygous *Tsc2* male mice (*Tsc2*$^{+/-}$) were bred with heterozygous *Fmr1* females (*Fmr1* x^{+}/x^{-}), both on the C57Bl/6J clonal background, to obtain F1 male offspring of four genotypes: WT (*Tsc2*$^{+/+}$, *Fmr1*$^{+/y}$), *Fmr1* KO (*Tsc2*$^{+/+}$, *Fmr1*$^{-/y}$), *Tsc2* Het (*Tsc2*$^{+/-}$, *Fmr1*$^{+/y}$) and cross (*Tsc2*$^{+/-}$, *Fmr1*$^{-/y}$) (Fig. 4b). All experimental animals were age-matched male littermates, and were studied with the experimenter blind to genotype and treatment condition. Animals were group housed and maintained on a 12:12 h light:dark cycle. The Institutional Animal Care and Use Committee at Massachusetts Institute of Technology approved all experimental techniques.

Electrophysiology. Acute hippocampal slices were prepared from P25-35 animals in ice-cold dissection buffer containing (in mM): NaCl 87, sucrose 75, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 25, $CaCl_2$ 0.5, $MgSO_4$ 7, ascorbic acid 1.3, and D-glucose 10 (saturated with 95% O_2/5% CO_2). Immediately after slicing, the CA3 region was removed. Slices were recovered in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 5, NaH_2PO_4 1.23, $NaHCO_3$ 26, $CaCl_2$ 2, $MgCl_2$ 1 and D-glucose 10 (saturated with 95% O_2/5% CO_2) at 32.5 °C for at least 3 h before recording.

Field recordings were performed in a submersion chamber, perfused with ACSF (2–3 ml min^{-1}) at 30 °C. fEPSPs were recorded in CA1 stratum radiatum with extracellular electrodes filled with ACSF. Baseline responses were evoked by stimulation of the Schaffer collaterals at 0.033 Hz with a two-contact cluster electrode (FHC) using a 0.2 ms stimulus yielding 40–60% of the maximal response. Field potential recordings were filtered at 0.1 Hz to 1 kHz, digitized at 10 kHz and analysed using pClamp9 (Axon Instruments). The initial slope of the response was used to assess changes in synaptic strength. Data were normalized to the baseline response and are presented as group means ± s.e.m. LTD was measured by comparing the average response 55–60 min after DHPG application to the average of the last 5 min of baseline.

The input–output function was examined by stimulating slices with incrementally increasing current and recording the fEPSP response. Paired-pulse facilitation was induced by applying two pulses at different inter-stimulus intervals. Facilitation was measured by the ratio of the fEPSP slope of stimulus 2 to stimulus 1. NMDAR-dependent LTD was induced by delivering 900 test pulses at 1 Hz. mGluR-LTD was induced by applying R,S-DHPG (50 μM) or S-DHPG (25 μM) for 5 min, or by delivering 1,200 pairs of pulses (with a 50 ms interstimulus interval) at 1 Hz. In some experiments, slices were incubated with the protein synthesis inhibitor cycloheximide (60 μM) for 30 min as follows: 20 min during baseline recording, 5 min during DHPG application and 5 min after DHPG application. For mGluR5 PAM experiments, slices were pretreated with CDPPB (10 μM) or DMSO control for 30 min in same manner as above, either in the presence of cycloheximide or control ACSF. For rapamycin experiments, slices were pretreated with rapamycin (20 nM) or DMSO control, with or without cycloheximide, for at least 30 min before recording and throughout the entire experiment. Significance was determined by two-way ANOVA and *post hoc* Student's *t*-tests. Statistics were performed using each animal as an '*n*', with each animal represented by the mean of one to four slices. All experiments were performed blind to genotype and included interleaved controls for genotype and treatment.

Metabolic labelling of new protein synthesis. Performed as described by Osterweil *et al.*[23]. Briefly, 500 μm slices were recovered for 4 h in 32.5 °C ACSF (in mM: 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 dextrose, 1 $MgCl_2$, 2 $CaCl_2$, saturated with 95% O_2 and 5% CO_2), incubated for 30 min with 25 μM ActD ± rapamycin (20 nM) or CDPPB (10 μM), and transferred to fresh ACSF ± drug with 10 μCi ml^{-1} [^{35}S]Met/Cys (Perkin Elmer) for another 30 min. After labelling, slices were homogenized, and labelled proteins isolated by TCA precipitation. Samples were read with a scintillation counter and subjected to a protein concentration assay (Bio-Rad). Final data were expressed as counts per minute per microgram of protein, normalized to the [^{35}S]Met/Cys ACSF used for incubation and the average incorporation of all samples analysed in that experiment. For autoradiography, homogenized slices were processed for SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose, stained for total protein using a Memcode staining kit (Pierce) and ^{35}S-incorporated proteins visualized with the aid of a phosphorimager (Fujifilm).

Immunoblotting. Immunoblotting was performed according to established methods using primary antibodies to Arc (Synaptic Systems), p-ERK1/2 (Thr 202/Tyr 204) (Cell Signaling Technology) or ERK1/2 (Cell Signaling Technology). ERK1/2 phosphorylation was measured by densitometry (Quantity One), and quantified as the densitometric signal of p-ERK1/2 divided by the ERK1/2 signal in the same lane. To quantify Arc expression, the densitometric signal of Arc was divided by the total protein signal (determined by Memcode staining) in the same lane.

Immunoprecipitation. Hippocampal slices (five to eight per animal) were metabolically labelled with 50 μCi ml^{-1} [^{35}S]Met/Cys for 3 h, and immunoprecipitation performed on yoked WT and *Tsc2*$^{+/-}$ slices essentially as described previously[23]. Briefly, slices were homogenized in immunoprecipitation lysis buffer (Pierce) plus protease inhibitors (EMD Biosciences), spun at 16,000*g* and supernatants pre-cleared with protein A/G sepharose. To avoid contamination of the Arc signal with IgG heavy chain, immunoprecipitation was performed using columns of monoclonal Arc antibody (a gift from P. Worley) crosslinked to protein A/G sepharose (Pierce Crosslink IP Kit). Immunoprecipitated Arc was resolved on SDS–polyacrylamide gel electrophoresis gels, transferred to nitrocellulose and exposed to a phosphorimager screen for 2–3 weeks. The same membranes were then immunoblotted for Arc. For each sample, the ratio of ^{35}S-incorporated: total was calculated by dividing the density of the band seen by autoradiography to the density of band seen by immunoblot (in the same lane).

Contextual fear conditioning. Six- to 12-week-old WT, *Tsc2*$^{+/-}$, *Fmr1*$^{-/y}$ and cross (*Tsc2*$^{+/-}$ × *Fmr1*$^{-/y}$) mice were fear conditioned to the training context with one 0.8 mA shock (2 s) as described by Ehninger *et al.*[4]. The mice were allowed 3 min to explore context before conditioning and were removed 15 s after the shock was given and returned to the home cage. Conditioned fear response was assessed 24 h later by a trained observer blind to condition, measuring the percentage of time spent freezing during the test period (3 min session). To determine context specificity of the conditioned response, mice trained at the same time were separated into two groups: one group was tested in the same training context, the other tested in a novel context. This novel context was created by varying distal cues, odour (2% acetic acid compared with 70% ethanol), floor material (plastic compared with metal bars) and lighting (red compared with white) of the testing apparatus. For rescue experiments, animals received a single injection of CDPPB (10 mg kg^{-1}, intraperitoneal) 30 min before training session.

Reagents: R,S-DHPG was purchased from Tocris Biosciences and S-DHPG was purchased from Sigma. Fresh bottles of DHPG were prepared as a 100× stock in H_2O, divided into aliquots and stored at −80 °C. Fresh stocks were made once a week. Rapamycin (EMD Biosciences) was prepared at 10 mM stock in DMSO and stored at −80 °C. The final concentration of rapamycin was 20 nM in less than 0.01% DMSO. Cycloheximide (Sigma) was prepared daily at 100× stock in H_2O. For slice experiments, CDPPB (EMD Biosciences) was prepared daily at 75 mM stock in DMSO with 0.5% bovine serum albumin (BSA) and diluted in ACSF to achieve a final concentration of 10 μM in less than 0.1% DMSO. For *in vivo* experiments, CDDPB was suspended in a vehicle consisting of 20% (2-hydroxypropyl)-(R)-cyclodextrin in sterile saline. All other reagents were purchased from Sigma.